Università degli Studi di Torino

PhD Programme in Biological Sciences
and Applied Biotechnologies

The Effects of Human Impacts on Cave Ecosystems and Subterranean Biodiversity

Tutor:
Marco Isaia

Candidate:
Giuseppe Nicolosi

XXXV° Cycle: 2019 /2022

Abstract

Subterranean ecosystems are considered relatively more vulnerable than other ecosystems due to their high stability relative to their surrounding surface environments. They are susceptible to numerous impacts often related to human activities, which make arduous the persistence of sensitive animal populations. Moreover, the most specialized organisms living therein are generally restricted geographically and numerically rare, making them vulnerable to even relatively minor disturbances.

As fragile and confined environments, caves are susceptible to numerous human impacts, such as indirect ones deriving from surface activities or direct ones, mostly related to the human presence in the cave. These threats include universal threats such as global warming, whereas others should be recognized as more regional or local threats.

The thesis aims to explore some of the main threats affecting subterranean habitats by developing different case studies at a different scale in both karst and volcanic caves, encompassing both emerging and unexplored threats. The main threats considered in this work are (i) the recreational use of caves, (ii) urbanization, agricultural and industrial activities, (iii) global climate change, and (iv) the presence of non-native species introductions.

The threat related to the “recreational use of caves” was investigated across different taxa at the ecosystem scale in three different case studies. Firstly, we tested the effect of the artificial lighting regime in two Italian Show Caves located in the Liguria region. According to our results, the duration of artificial illumination in caves significatively influences the growth of lampenflora – i.e., photosynthetic microorganisms forming biofilms on cave speleothems, causing structural and aesthetic damage – especially in show caves experiencing high touristic use. Second, we tested whether the presence of visitors may affect the abundance of cave-dwelling arthropods, using the isopod *Armadillidium lagrecai* as a model organism in the semi-touristic Monello karst cave, in Sicily. The species abundance seems to solely increase with increasing temperature and humidity with no effect due to the presence of visitors. Third, we provided a first insight into the microbial diversity of the Etna volcano lava tubes and revealed the presence of a highly diversified microbial community, including a number of taxa related to the anthropic disturbance.

Regarding “urbanization, agricultural, and industrial activities” we showed that the local and unique biological assemblage living in Monte Conca sinkhole (Sicily) supported by an autochthonous food source, is highly threatened by surface agricultural activities, which are causing drastic alteration of the seasonal water regime, also determining the entrance of several contaminants of anthropogenic origin.

As for “global warming”, future predictions developed by SDM to predict remarkable impacts at the regional scale on the survival of the cave-dwelling spider *Meta menardi*, which was documented on Mount Etna for the first time.

Finally, we reviewed the threat of “non-native species introductions” globally by means of bibliometric analysis. The literature search revealed the presence of 246 alien species listed in the literature encompassing several taxa. Our data suggested that only a limited number of alien species may represent a real threat to subterranean ecosystems and to the species living therein, probably due to the strong ecological filter imposed by subterranean systems. However, future studies are recommended to deepen the understanding of invasiveness in subterranean habitats.

Despite the growing literature concerning the subterranean biome and the extent of potential threats, our results revealed that some threats result still largely unexplored. Future investigations are recommended to provide practical suggestions and future directions for management practices.

Keywords: biospeleology, subterranean ecosystems, threat, lampenflora, climate change, alien species, human contamination, conservation biology

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This thesis is based on the following original publications and manuscripts, referred to by Roman numerals in the text.

Paper I

Piano, E., **Nicolosi, G.**, & Isaia, M. 2021. Modulating lighting regime favours a sustainable use of show caves: A case study in NW-Italy. *Journal for Nature Conservation*, 64(3):126075

Paper II

Nicolosi, G., Mammola, S., Costanzo, S., Sabella, G., Cirrincione, R., Signorello, G., & Isaia, M. 2021. Microhabitat selection of a Sicilian subterranean woodlouse and its implications for cave management. *International Journal of Speleology*, 50(1):53-63.

Paper III

Nicolosi, G., Gonzalez-Pimentel, J.L., Piano, E., Isaia, M. & Miller, A.Z. 2023. First insights into the microbial diversity of Mount Etna volcanic caves. *Microbial Ecology*, 1-14.

Paper IV

Nicolosi, G., Galdenzi, S., Messina, M. A., Miller, A. Z., Petralia, S., Sarbu, S. M., & Isaia, M. 2022. Sulfidic Habitats in the Gypsum Karst System of Monte Conca (Italy) Host a Chemoautotrophically Supported Invertebrate Community. *International Journal of Environmental Research and Public Health*, 19(5), 2671.

Paper V

Nicolosi, G., Piano, E. & Isaia, M. 2023. Habitat preference of *Meta menardi* and *M. bourneti* (Araneae, Tetragnathidae) along the altitudinal gradient of Mount Etna (Sicily, S–Italy). *Journal of Arachnology* (*accepted*).

Paper VI

Nicolosi, G., Piano, E. & Isaia, M. Future shifts of cave-dwelling *Meta* spiders under the influence of global warming on Etna volcano, Sicily. *Manuscript in preparation*.

Paper VII

Nicolosi, G., Mammola, S., Verbrugge, L. & Isaia, M. 2023. Aliens in caves: the global dimension of biological invasions in subterranean ecosystems. *Biological Reviews (Early View).*

1. Introduction

1.1. General overview

Subterranean ecosystems are among the most widespread environments on Earth. The most well-known among these are caves, but from a modern and generally accepted perspective, the subterranean domain is much more extensive, encompassing both air- and water-filled underground habitats.

Although subterranean habitats are more widespread and diversified than is usually perceived, we still know too little about subterranean biota due to the intrinsic inaccessibility of subterranean ecosystems (Ficetola et al, 2019) and many impediments which make research activities arduous (Mammola et al., 2021).

All subterranean habitats, whether they have large or small spaces, share some peculiar environmental characteristics (Culver & Pipan, 2009). They completely or almost completely lack photosynthetic productivity, being supported either by inputs of organic matter from the surface or by in-situ sources of inorganic chemical energy. As a result, the majority of caves have very low energy and nutrient availability and thus, extremely low biological activity and productivity.

Moreover, environmental variability is very reduced when compared to surface habitats. Most subterranean systems have a nearly constant temperature over the year and a relative humidity often constantly close to saturation (Badino, 2010; Cigna, 2002). The thermic excursions progressively decrease toward the innermost cave sections, until stability is reached at the value corresponding to the average annual value of the outside temperature (Moore and Nicholas 1964), at least in temperate climates.

Subterranean organisms exhibit numerous adaptations to cope with the peculiar environmental conditions of their subterranean habitat. They show specific morphological and behavioral adaptations such as the loss or reduction of eyes and pigmentation, longer appendages and enhancements of nonvisual sensory systems. They have also evolved physiological characteristics, most notably reduced energy consumption, which has a high selective advantage in cave animals and has been observed in numerous species in a variety of phyla (Poulson, 1963; Culver, 1982; Hüppop, 2000; White & Culver, 2011). Other biological factors include low reproductive rates, high susceptibility to environmental change, and the reduced ability to respond to environmental stress. In light of these biological attributes, subterranean habitats and the species living therein are theoretically vulnerable even to minor changes in the environment.

1.2. Main threats to subterranean habitats and biodiversity

Despite a recent increase in interest and concern in subterranean ecosystems and their specialized fauna, they are still only superficially known. Most of the threats affecting the subterranean domain were highlighted by Mammola and colleagues (2019a) in their manifesto.

Being fragile and confined environments, caves are relatively more vulnerable than other ecosystems (Elliott, 2000; Hamilton-Smith & Eberhard, 2000). Moreover, the most specialized organisms living therein are generally restricted geographically and are numerically rare, making them vulnerable to even relatively minor disturbances (Culver & Pipan, 2009). They are susceptible to numerous impacts often related to human activities, which further threaten the persistence of sensitive animal populations (Culver & Pipan, 2019; Romero, 2009). These threats include both general threats such as global warming and regional or local threats.

Surface impacts can indirectly reach subterranean ecosystems and contribute to subterranean habitat degradation. These can cause irreversible damage to subterranean ecosystems. They encompass, among others, (i) the habitats degradation due to urbanization, industrial and agricultural activities (Trajano, 2000; Reboleira et al., 2011; Souza-Silva et al., 2015; Sugai et al., 2015), (ii) the rise of temperature caused by global climate change (Mammola et al., 2018, 2019), and (iii) the introduction of non-native alien species that compete with or prey upon native subterranean populations (Howarth et al., 2007; Wynne et al., 2014).

Other impacts can directly affect the subterranean habitats and are often related to human use of caves. These threats encompass (iv) the variation of microclimatic parameters due to the intensive use caves for recreation and tourism (Isaia et al., 2011; Pellegrini & Ferreira, 2012, 2016; Alonso et al., 2019; but see Faille et al., 2014), (v) the presence of artificial lighting and tourism infrastructure (i.e., paving paths, creating additional entrances, altering air circulation) (Isaia et al., 2011; Mulec, 2019; Piano et al., 2015), and (vi) the introduction of pathogenic microorganisms by visitors (Chelius et al., 2009; Mammola et al., 2017; Saiz-Jimenez, 2012; Zhelyazkova et al., 2020).

1.2.1. Recreational use of caves

Human-induced impacts associated with touristic caves can affect different ecosystem components (Mammola, 2019a).

The joint effect of visitors and artificial lights directly influences the presence and distribution of microorganisms. For instance, organic materials passively transferred into caves by visitors enhance the growth of bacterial and fungal species alien to the cave community (Chelius et al., 2009; Mammola et al., 2017; Pfendler et al., 2018).

The installation of artificial lights leads to the development of photosynthetic biofilms on cave rocky surfaces, i.e. the so-called ‘lampenflora’ (Baquedano Estevez et al., 2019; Cigna, 2011; Falasco et al., 2014; Mulec et al., 2008; Mulec, 2019; Piano et al., 2015).

Lampenflora mainly consists of prokaryotic and eukaryotic microorganisms of various taxonomic groups including Cyanobacteria, diatoms (Bacillariophyta), green algae (Chlorophyta) and, in later successional stages, bryophytes, ferns and vascular plants (Castello, 2014; Mulec & Kubesova, 2010).

The lampenflora growth represents a severe threat to the subterranean environment due to its aesthetic impact (Mulec & Kosi, 2009), deterioration of the substrate (biocorrosion) (Albertano et al., 2003), and physical alterations including structural changes and disintegration of the substrate through mechanical pressure (Caneva et al., 2008; Warscheid & Braams, 2000). In addition, dead masses generated by biofilms introduce a substantial amount of nutrients that may become available to consumers, likely favoring opportunistic species of bacteria and fungi (Baquedano-Estevez et al., 2019).

Quantifying the effect of the main environmental drivers favoring lampenflora or, more practically, controlling the growth of photosynthetic biofilms is thus a primary concern of show cave managers (Grobbelaar, 2000; Mulec, 2014).

A massive tourist presence may directly alter the underground microclimate and result in seasonal variations in temperature and relative humidity (Cigna, 2004). Considering the vulnerability of subterranean invertebrates even to minor changes in the environment, microclimate modifications associated with tourism can potentially contribute to the extirpation of the local fauna (Juberthie, 2000; Slaney & Weinstein, 1997; Krajick, 2001; van Beynen & Townsend, 2005). A greater knowledge of the main physical parameters characterizing the cave (in particular temperature, humidity and CO2) is essential for the conservation of the cave environment and its fauna.

1.2.2. Urbanization, agricultural, and industrial activities

Subterranean ecosystems are generally regarded as oligotrophic environments in term of energy inputs, especially in the inner and most isolated areas (Culver & Pipan, 2019). Nutrient supplies mainly depend on the flux of energy from the surface, which enters the subterranean habitats in a variety of ways: carried by gravity from the cave entrances and ceiling cracks, by water, wind, or by animals such as bats, birds or rodents (Gibert et al., 2002; Simon et al., 2003; Hüppop, 2005; Fenolio et al., 2006; Fišer, 2019).

Several subterranean ecosystems do not depend entirely on organic matter coming from the surface (Sarbu et al., 1996; Stevens, 1997; Galdenzi & Sarbu, 2000; Krumholz, 2000; Por et al., 2013; Popa et al., 2019; Brad et al., 2021) but instead on chemoautotrophic in-situ production of organic matter by microorganisms processing different inorganic elements to obtain energy and nutrients. These organisms are the only primary producers inhabiting the subterranean ecosystem, but any alteration of their environment may lead to severe and irreversible disturbance of these exceptional ecosystems.

Human changes to the surface environment will alter the equilibrium, function and diversity of subterranean microbial communities (e.g., Davis et al., 2020) as well as the water regime (Čučković, 1983; Minanović, 1990).

Specifically, agricultural and industrial activities, especially the extensive use of irrigation in agriculture, have become an increasing concern in areas featuring karst bedrock. The alteration of the water regime, the oversupply of nutrients and the input of bacteria can lead to algal growth, oxygen depletion, and, consequently, negatively affect fragile subterranean ecosystems and the organisms inhabiting them (e.g., Lory, 1999; Davis et al., 2020; Danielopol et al., 2003).

1.2.3. Global climate change

Anthropogenic climate change is impacting biological systems at multiple scales in terrestrial and marine ecosystems (e.g. Parmesan & Yohe, 2003; Harley et al., 2006; Brierley & Kingsford, 2009). Recent studies have used isolated ecosystems as models for unraveling ecological responses to recent climate alterations, extrapolating the results and conclusions to a wider range of systems and organisms (Mammola et al., 2019b; Whittaker et al., 2017). The use of hypogean environments to explore the effect of climate change on biological communities has recently increased in frequency (e.g., Wynne et al., 2014; Sánchez-Fernández et al., 2016; Mammola et al., 2018; Mammola et al., 2019b).

Being extremely stable relative to their surrounding external environments (Poulson & White, 1969; Howarth, 1993) subterranean ecosystems - and the species living therein - often show pronounced sensitivity to even small changes in their environmental conditions (e.g., Barr & Kuehne, 1971; Howarth, 1980; Mammola et al., 2019c).

A rise in average global temperature will also increase average temperatures of subterranean habitats. This change may be lethal for some subterranean species, whose coping mechanisms may be limited to shifting their distributional range (Mammola & Isaia, 2017).

This issue is particularly important for low-dispersal animals such as hypogean arthropods which have reduced physiological adaptability (e.g., Cardoso, 2012; Novak et al., 2014; Mammola et al., 2015). Cave-dwelling invertebrates with poor subterranean adaptations have conversely retained the ability to disperse effectively outside the caves in certain phases of their development and may potentially shift their distribution towards more climatically suitable areas.

1.2.4. Non-native species introductions

Alien species are a serious threat to native species and ecosystems (Pyšek et al., 2020). Despite global efforts, invasive species are rapidly increasing worldwide (Meyerson & Mooney, 2007).

The effect of biological invasions may be difficult to quantify and the magnitude of the impact may significantly vary across different ecosystem types (Vilà et al., 2010, 2011; Diagne et al., 2021). Furthermore, some habitats may be less prone to biological invasions than others due to their abiotic and biotic characteristics and only invaders possessing traits closely related to native organisms may be successful (Pyšek et al., 2009, 2010).

Subterranean ecosystems are quintessential examples of habitats with strong environmental filters (e.g., lack of light and scarcity of food), selecting for convergent adaptations in species that have successfully adapted to life in darkness. Despite these stringent environmental constraints, the number of records of alien species in subterranean ecosystems has increased in the last decades, but the literature on the subject remains largely fragmented and mostly anecdotal, and the susceptibility to impacts by alien species remains untested for all but very specific cases.

Exotic species can certainly represent important threats to endemic fauna in epigean habitats as several invasive exotic troglophiles are capable of invading cave habitats and might even competitively exclude troglobitic species (Revees, 1999), but in-depth studies are urgently needed to shed light on the true effect of the presence of alien species in these habitats.

2. Objectives

Understanding the ways in which a particular threat affects subterranean ecosystems would aid in prioritizing the most important and/or most manageable threats.

Threat ranking and prioritization will depend on the scale at which the threats are found; thus, quantifying and understanding the main drivers of biological responses to human disturbances at multiple scales is foundational to effective conservation plans and management systems.

For instance, a large-scale threat (e.g., climate change) must primarily be addressed at regional to global scales, whereas threats such as tourist activities require local to regional management.

In this regard, this thesis aims to explore some of the main threats affecting subterranean habitats in both karst and volcanic caves in order to provide a wider understanding of their response to anthropogenic pressure. It also aims to identify important gaps in knowledge and areas for future research and prioritization.

The thesis will investigate several case studies at different scales, focused on the following threats:

I. The recreational use of caves (i.e., cave tourism): **Paper I, II, III**
II. Urbanization, agricultural, and industrial activities: **Paper IV**
III. Global climate change: **Paper V, VI**
IV. The presence and impact of non-native species: **Paper VII**

The workflow used in this PhD thesis is presented in Figure 1.

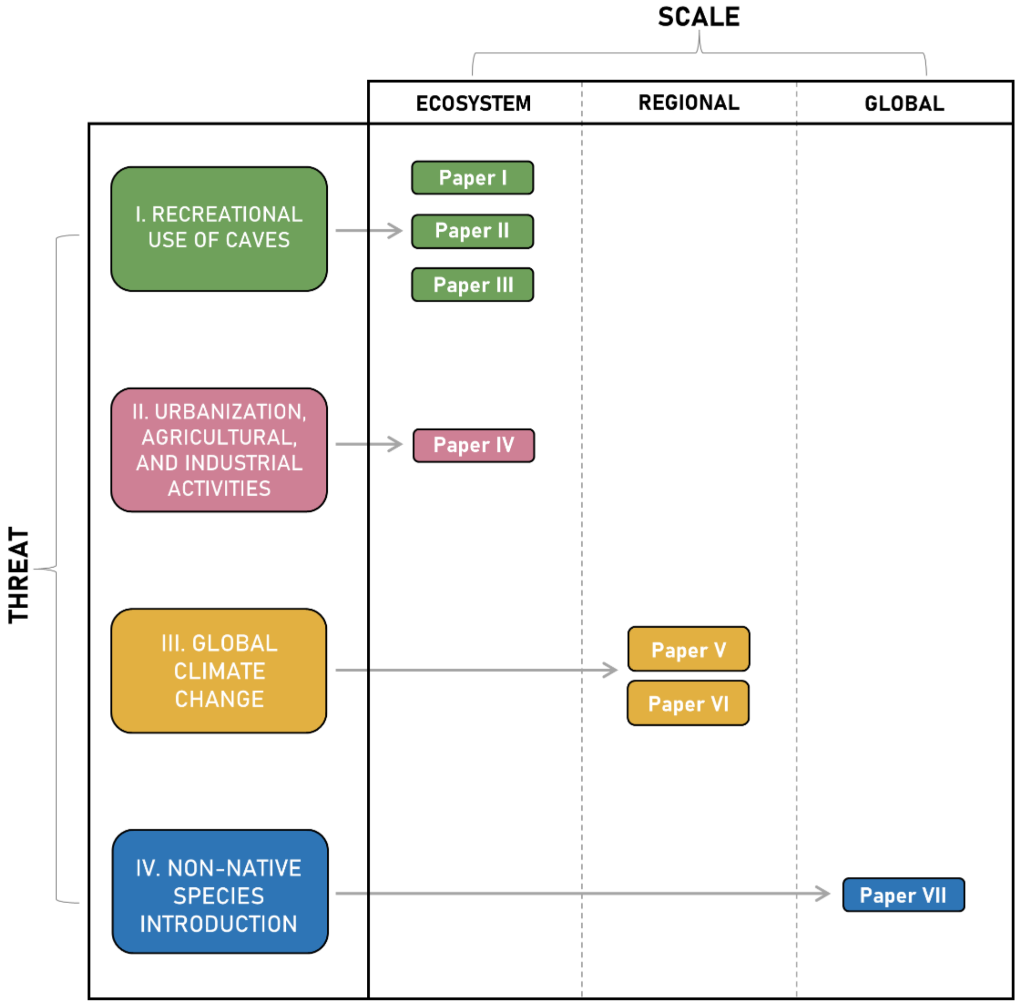

Figure 10. Workflow between the main threats to the subterranean realm here considered and respective papers developed at the ecosystem, the regional and the global scale.

The threat of “**recreational use of caves**” was investigated in **Papers I, II, III**. The response to this threat was compared across different taxa at the **ecosystem scale**.

Paper I aims to quantify the main environmental drivers favoring the proliferation of photosynthetic organisms in show caves – i.e., lampenflora. The effect of both the lighting regime and environmental parameters was simultaneously tested to identify the most vulnerable areas in show caves.

Paper II explored the relative influence of microclimatic variations and the presence of visitors on the spatio-temporal dynamics of specialized subterranean fauna. We used the Monello Cave (a karst system located in a Strict Natural Reserve in eastern Sicily) as a model system. Monello Cave is renowned for its peculiar subterranean terrestrial fauna (Caruso, 1994). In particular, we focused on the specialized subterranean woodlouse *Armadillidium lagrecai* Vandel (Malacostraca: Isopoda: Armadillidiidae) and its response to both natural and anthropogenic factors related to cave tourism.

Paper III provided the first microbiological assessment of biofilms coating the walls of lava tubes located in Mount Etna Park (Sicily, Italy). The investigation aims to highlight the uncharted microbial diversity of Mount Etna lava caves, and contribute to the preservation of these unique geoheritage sites.

The threats of "**urbanization, agricultural, and industrial activities**" were investigated at the **ecosystem scale** in **Paper V**. The biological community dwelling in the Monte Conca sinkhole (W-Sicily, Italy), a karst system frequently affected by surface water inputs derived from agricultural activities, was described. The paper elucidates the role of the resident chemoautotrophic organisms in the cave food web, providing evidence for their independence from external inputs.

The threat of "**global climate change**" was explored at the **regional scale** in **Papers V** and **VI**. The information collected in Paper V was also used in Paper VI. Paper VI investigates the environmental factors explaining the niche segregation of the cave dwelling spiders *Meta menardi* and *M. bourneti* on Mount Etna, in order to predict the future changes in species distribution under a global warming scenario.

The threat of "**non-native species introductions**" was investigated at a **global scale** in **Paper VII** by performing a bibliometric analysis on the topic. A literature review and synthesis was conducted to identify current knowledge on alien species detected in subterranean habitats. The review focused on global terrestrial and freshwater subterranean habitats and investigated the state of the knowledge on alien species origin, destination, and their environmental and socio-economic impacts in subterranean ecosystems. Moreover, we tested the relationship between the presence/absence of adaptive traits facilitating the colonization and establishment of a species in the subterranean environment.

3. Material and Methods

3.1. Study areas

Sampling activities were performed in Southern Italy (Sicily) and in North-western Italy (Fig. 2).

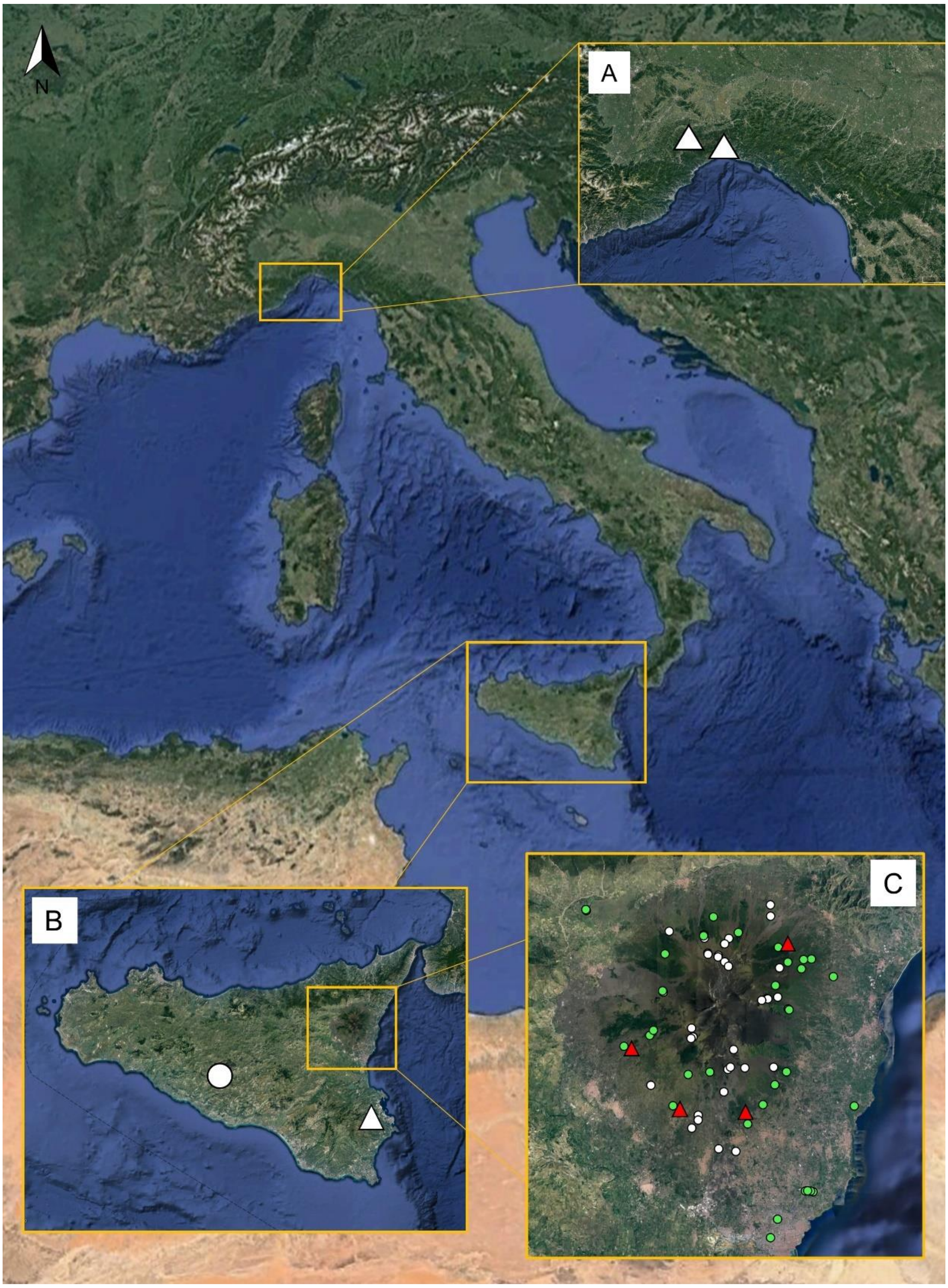

Figure 11. A map of site locations. Location of the sites used in Paper I (triangle) (**A**); location of the sites used in Paper II (triangle) and Paper IV (circle) (**B**); Location of the sites used in Paper III (red triangle), Paper V (white circles) and Paper VI (green and white circles) (**C**).

Data for Paper I was collected in two Italian Show Caves located in the Liguria region (NW-Italy). The caves were 15 km apart (Fig. 2A). The study for Paper II was conducted in the municipality of Syracuse (southeastern Sicily), in the karst system of Monello Cave (Fig. 2B). The cave is within the boundaries of the Strict Nature Reserve “Grotta Monello”. The bacterial communities of four lava caves of Mount Etna were investigated in Paper III (Fig. 2C). Finally, the occurrence of spiders in the genus *Meta* were investigated in several lava caves of Mount Etna in Papers V and VI (Fig. 2C).

3.2. Threat I: Recreational use of caves

3.2.1. The impact of cave lighting on show caves (Paper I)

In Paper I, we evaluated the presence and concentration of the lampenflora along the tourist path of Toirano cave (high number of visitors) and Valdemino cave (low number of visitors). We selected 22 and 14 plots in each area, respectively (i.e., illuminated speleothem) located on average every 50 m from the cave entrance along the tourist path. Here we measured the density of cyanobacteria, green algae and diatoms, the three main groups constituting the photosynthetic component of biofilms in caves. We then characterized the microhabitat by taking environmental measures including light intensity, water content, and distance from the lamp.

We used Generalized Linear Models (Zuur et al., 2009) to determine the relationship between predictor variables (light intensity, distance from the cave entrance, water presence and tourist use) and dependent variables (total chlorophyll-a concentration, and the chl-a concentrations of cyanobacteria, diatoms and green algae).

3.2.2. Factors affecting the abundance of subterranean fauna (Paper II)

In Paper II, we explored the influence of human disturbance on the spatio-temporal dynamics of the specialized isopod *Armadillidium lagrecai*. We focused on Monello cave, a karst system recently opened to a limited number of visitors.

We set the number of visitors and the distance from the tourist path as proxies for human disturbance based on previous studies (Faille et al., 2014; Piano et al., 2015; Mammola et al., 2017).

We assigned to each sampling event the total number of visitors to the cave in the previous two weeks. Using this method, we selected nine sampling points along the visitor pathway, and eight points further away from the path and/or in the lateral branches not opened to public. We measured the abundance of *Armadillidium lagrecai* at each sampling session using three sheets of corrugated cardboard. We then characterized the microhabitat at each sampling point by collecting information on distance from the cave entrance and from the pathway, temperature and relative humidity.

We then tested the abundance of *A. lagrecai* against the predictor variables: temperature (measured as standard deviation of temperature values during the whole sampling period), subjacency (vertical distance from the surface), the sampling week (cumulative counting), distance from the tourist path, and the number of visitors.

The temporal distribution of *A. lagrecai* was modeled using a generalized additive model (Hastie, 1991) due to *A. lagrecai* abundance demonstrating a non-linear response to the sampling week. The effect of microclimatic conditions and tourism on the abundance of *A. lagrecai* was analysed separately (to avoid the confounding effect of seasonality) by using a generalized linear mixed model (Zuur et al., 2009).

3.2.3. Taxonomic characterization of microbial communities on Etna lava caves (Paper III)

A first microbiological assessment of biofilms coating the walls of lava tubes of Mount Etna Park (Sicily, Italy) was the main subject of Paper III. Four lava tubes were sampled. We sorted the four caves according to tourist use (“Grotta Catanese I” and "Grotta di Monte Corruccio” were visited occasionally by a small number of tourists and “Grotta del Santo” and “Grotta Lunga” were visited more frequently).

Samples of colored microbial mats were aseptically collected using sterile scalpels and stored in sterile 1,5 mL microtubes at 4 °C until transportation to the lab, then stored at −80 °C until laboratory procedures were performed.

Samples were then examined by field emission scanning electron microscopy (FESEM) to characterise microbial morphology.

Identification of the microbial communities present in the lava tube were analysed using molecular biology techniques based on 16S rRNA gene analysis.

Taxonomic identification was carried out using SILVA database v.132. Alpha diversity metrics (Shannon’s Diversity Index and Pielou’s evenness) of bacterial communities were also calculated to investigate community heterogeneity within sample diversity.

3.3. Threat II: Urbanization, agricultural, and industrial activities: Paper IV

The threats of “urbanization, agricultural, and industrial activities” were explored in the gypsum cave of Monte Conca, a sinkhole whose ecological equilibrium is threatened by the entrance of water from anthropogenic inputs (i.e., agricultural activities around the Monte Conca sinkhole) (Davis et al., 2020). Here, a sulfidic spring is located in the lower gallery.

To shed light on the biological assemblages living in the Monte Conca sinkhole and to promote its protection, the species composition of the invertebrate community and the trophic interactions among organisms were investigated in both sulfidic and non-sulfidic habitats.

The faunal investigation was conducted visually and by using hand sampling methods during the dry the wet seasons. Presence/absence data was collected on the aquatic and terrestrial communities.

Stable Isotope Ratio Analysis (SIRA) of carbon and nitrogen was used to determine the level of independence from the surface food web (Sarbu et al. 1996). SIRA represents an effective tool to determine food sources in a given ecosystem because organisms fractionate isotopes of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{13}N$) in predictable ways. The invertebrates were collected in the dry season i.e., when maximal isolation from the surface is achieved. Samples were assigned to the "sulfidic habitat" category when collected within or in proximity to the sulfidic pools, and to the "non-sulfidic habitat" when collected within or in close proximity to the control pool. Analyses were performed on the most detectable species. Moreover, we collected a sample of the white biofilm in the sulfidic pool as this could represent a food source for a chemoautotrophically-based ecosystem. For comparison, a sample of organic matter was also collected in the non-sulfidic habitat (i.e., decaying plant matter and other organic remains of surface origin).

3.4. Threat III: Global climate change: Paper V, VI

3.4.1. Sampling and habitat requirements of *Meta* spiders (Papers V)

Papers V and VI investigated the effect of climate change on the population of *Meta* spiders along the altitudinal gradient of Mount Etna. We surveyed 27 caves opening at different altitudes and on different slopes of the volcano. The presence of *Meta* spiders at each site was verified through visual census and occasional hand sampling.

The environmental preferences of *Meta* spiders living in Mount Etna lava caves were defined in terms of annual mean temperature, annual precipitation, elevation, and land use using the nonparametric MANN–WHITNEY test for non–normal distributed variables.

3.4.2. Niche hypevolume of *Meta* spiders (Paper VI)

Using data retrieved in Paper V on ecological factors determining the segregation of the two species of *Meta* spiders detected on the volcano slopes, we identified the land use in a 50m radius around each sampling site using QGis (Quantum Gis Development, 2022). Land use was obtained from regional data (www.sitr.regione.sicilia.it) and we calculated the percentage of the following three land use categories: vegetated areas, lava fields and anthropogenic use.

Bioclimatic data for the years 1970-2000 and 2070 were obtained from the WorldClim website (www.worldclim.org) with a spatial resolution of 30 seconds.

Collinearity among bioclimatic variables were reduced by performing a Principal Component Analysis (PCA). We then tested for multicollinearity among the PCA axes and land use variables with the R Pearson test. As a results, only the three PCA axes were retained for the subsequent analysis of the hypevolumees sensu (Hutchinson 1957).

3.4.3. Modelling the future distribution of *Meta* spiders (Paper VI)

The distribution of *Meta bourneti* and *M. menardi* in Sicily were predicted for future scenarios (2070) under a high-emission scenario (RCP) 8.5 (van Vuuren et al. 2011). The data are climate projections from the general circulation model (GCM) CCSM4 that were downscaled and calibrated (bias corrected) using WorldClim 1.4 as the baseline climate.

Choice of environmental predictors: The current and future distribution of the species *M. bourneti* and *M. menardi* spiders in the study area was performed using 19 bioclimatic variables and elevation data at a resolution of 2.50 (nearly 4.5 km at the equator) to represent current climatic conditions (1950–2000; Hijmans et al., 2005; resolution: 30''; www.worldclim.org).
After checking for collinearity, we reduced the full set of 19 environmental variables to 5 (BIO07 Temperature Annual Range, BIO08 Mean Temperature of Wettest Quarter, BIO16 Precipitation of Wettest Quarter, BIO18 Precipitation of Warmest Quarter, BIO19 Precipitation of Coldest Quarter).

Modelling procedures: presence-absence modelling was performed for both species using 3 commonly-used models in SDM ('sdm' package, Naimi & Araújo, 2016). Models included the Generalised Linear Model (GLM) (McCullagh & Nelder, 2019), the Boosted Regression Tree (BRT) (Elith et al., 2008), and the Flexible Discriminant Analysis (FDA) (Hastie et al., 1994). For modelling, the occurrence of one species at a given point was considered a true absence of the other species and vice versa.
The 'sdm' package was used to combine the distribution maps using the "ensemble" function to produce consensus maps based on weighted AUC values (Naimi & Araújo, 2016). Ensemble prediction is the process of integrating multiple individual models to provide robust estimates of potential species distributions (e.g., Araujo & New, 2007; Marmion et al., 2009; Meller et al., 2014; Poulos et al., 2012; but see Crimmins et al., 2013; Zhu & Peterson, 2017).

3.5. Threat IV: Non-native species introductions (Paper VII)

The threat "presence of non-native species introductions" was assessed in Paper VII using a bibliometric analysis on the occurrence of subterranean alien species in both terrestrial and freshwater subterranean habitats at global scale.
A systematic literature survey was conducted using Clarivate Analytics Web of Science and simultaneously on grey literature including technical reports, non-English articles (Haddaway et al., 2020; Chowdhury et al., 2022) and Google Scholar (Haddaway et al., 2015).
For each relevant paper, we read the full text and extracted detailed information. Moreover, we explored specialized literature to collect species-specific traits for each subterranean species in our database (presence/absence of adaptations commonly present in subterranean species in

accordance with Pipan & Culver (2012) in order to verify whether the species that successfully colonize subterranean environments display pre-adaptative traits.

Using a GLM (Bate et al. 2015), we tested the response variable “species established” or “species not established” (binary data) against predictor variables: adaptive traits (yes or no), trophic level (“Detritivore”, “Predator”, “Omnivore”, and “Others”) and presence of wings (proxy of dispersibility in a range-expanding population).

4. Results and Discussion

4.1. The impact of cave lighting on show caves (Paper I)

The impact of cave lighting on show caves was tested at the ecosystem scale in Paper I by comparing two show caves sharing similar environmental conditions and management, but different tourist use. The results of the GLM model highlighted that a higher tourist flux significantly enhances the overall proliferation of lampenflora as demonstrated by the higher overall chl-a concentrations present in Toirano cave (Table 1). Moreover, a positive effect of light intensity on the total chl-a concentration was highlighted. Accordingly, these two parameters represent the main drivers of the abundance of photosynthetic microorganisms in show caves.

As the light intensity measured within the two show caves was fully comparable, we were able to ascribe this positive effect uniquely to the longer periods of illumination in Toirano cave, i.e. where the tourist flux was higher.

The effect of the illumination length was consistent across the three groups when was considered the separate response of cyanobacteria, diatoms and green algae. All of them showed significantly higher chl-a concentrations in the cave subjected to the highest tourist flux (Table 1).

Besides the number of illumination hours, light intensity significantly increased the total chl-a concentration, confirming data obtained in literature (Mulec, 2014), but this effect was not consistent across the three groups, in contrast with Piano et al. (2015). In fact, the overall proliferation of the lampenflora was mainly driven by an increase in cyanobacteria and, partially, in diatoms, but not of green algae (Table 1). This discrepancy can thus assume that light intensity was below the minimum threshold to show a clear positive effect on the growth of eukaryotic algae, while the proliferation of cyanobacteria is favored even by light intensities below 10 lx (Albertano, 2012; Bruno & Valle, 2017).

The presence of water significantly influences lampenflora proliferation by favoring the growth of diatoms (Table 1). Accordingly, the presence of seeping water is expected to enhance the water reaction with limestone and the consequent release of Ca2+ ions, which are needed by diatoms to adhere to the rock surface (Mulec, 2012).

Finally, the establishment of lampenflora was not significantly influenced by any of the factors included in the statistical models (Table 1). It is therefore plausible that the occurrence pattern of the three examined photosynthetic microorganism groups is determined by a stochastic distribution as often occurs in microorganisms with passive dispersal (Mulec, 2019). After their installation, their growth is then strongly driven by the local environmental conditions, which may determine or not their extensive proliferation (Falasco et al., 2014; Martinez & Asencio, 2010).

Table 4. Estimated parameters (*β-est*), standard errors (*SE*), t- (*t*) or z-values (*z*) and p-values (*P*) for each covariate in the GLMs performed on the total chl-a (a), and in the ZIG models for each photosynthetic group, including: b) outputs of the binomial GLMs performed on presence/absence data; and c) outputs of the gamma GLMs on concentration values > 0 (the number of data used (n) is reported for each group). Significant results are highlighted in bold.

a)	***Variables***	***β-est***	***SE***	***t***	***P***
Total chl-a	*Touristic_use*	1.96	0.592	3.31	**0.002**
	Light_intensity	0.683	0.217	3.15	**0.004**
	Humidity	0.812	0.508	1.60	0.120
	Distance_entrance	-0.004	0.003	-1.46	0.155
b)	***Variables***	***β-est***	***SE***	***z***	***P***
Cyanobacteria	*Touristic_use*	0.114	0.574	0.198	0.843
	Light_intensity	-0.354	0.217	-1.63	0.103
	Humidity	-0.445	0.480	-0.927	0.354
	Distance_entrance	0.003	0.003	1.05	0.294
Diatoms	*Touristic_use*	-0.104	0.793	-0.131	0.896
	Light_intensity	0.991	0.817	1.21	0.225
	Humidity	-0.204	1.00	-0.204	0.839
	Distance_entrance	0.001	0.004	0.084	0.933
Green algae	*Touristic_use*	0.334	0.760	0.440	0.660
	Light_intensity	0.410	0.314	1.31	0.191
	Humidity	0.076	0.723	0.105	0.916
	Distance_entrance	-0.005	0.004	-1.25	0.211
c)	***Variables***	***β-est***	***SE***	***t***	***P***
Cyanobacteria	*Touristic_use*	1.84	0.564	3.26	**0.001**
(n = 28)	*Light_intensity*	1.01	0.234	4.31	**< 0.001**
	Humidity	-0.935	0.706	-1.33	0.185
	Distance_entrance	-0.001	0.002	-0.922	0.357
Diatoms	*Touristic_use*	1.25	0.381	3.27	**0.001**
(n = 33)	*Light_intensity*	0.401	0.167	2.40	0.059
	Humidity	0.965	0.385	2.50	**0.012**
	Distance_entrance	-0.002	0.002	-1.64	0.101
Green algae	*Touristic_use*	3.68	0.787	4.67	**< 0.001**
(n = 11)	*Light_intensity*	0.147	0.505	0.292	0.771
	Humidity	1.12	0.626	1.78	0.075
	Distance_entrance	-0.002	0.003	-0.775	0.438

4.2. Factors affecting the abundance of subterranean fauna (Paper II)

In Paper II, we investigated the effect of the presence of visitors and of environmental parameters on the abundance of cave-dwelling isopod *Armadillidium lagrecai*.

Results showed a significantly increase in species abundance with increasing temperature and relative humidity, with a peak at around 18°C and relative humidity approaching or reaching saturation (Fig. 3). Data suggested that microclimate influences the spatial distribution of *A. lagrecai*, confirming its preference for the warmest and most stable cave sections, as well as an apparent seasonality effect on abundance variation which is possibly related to reproduction (Caruso, 1994).

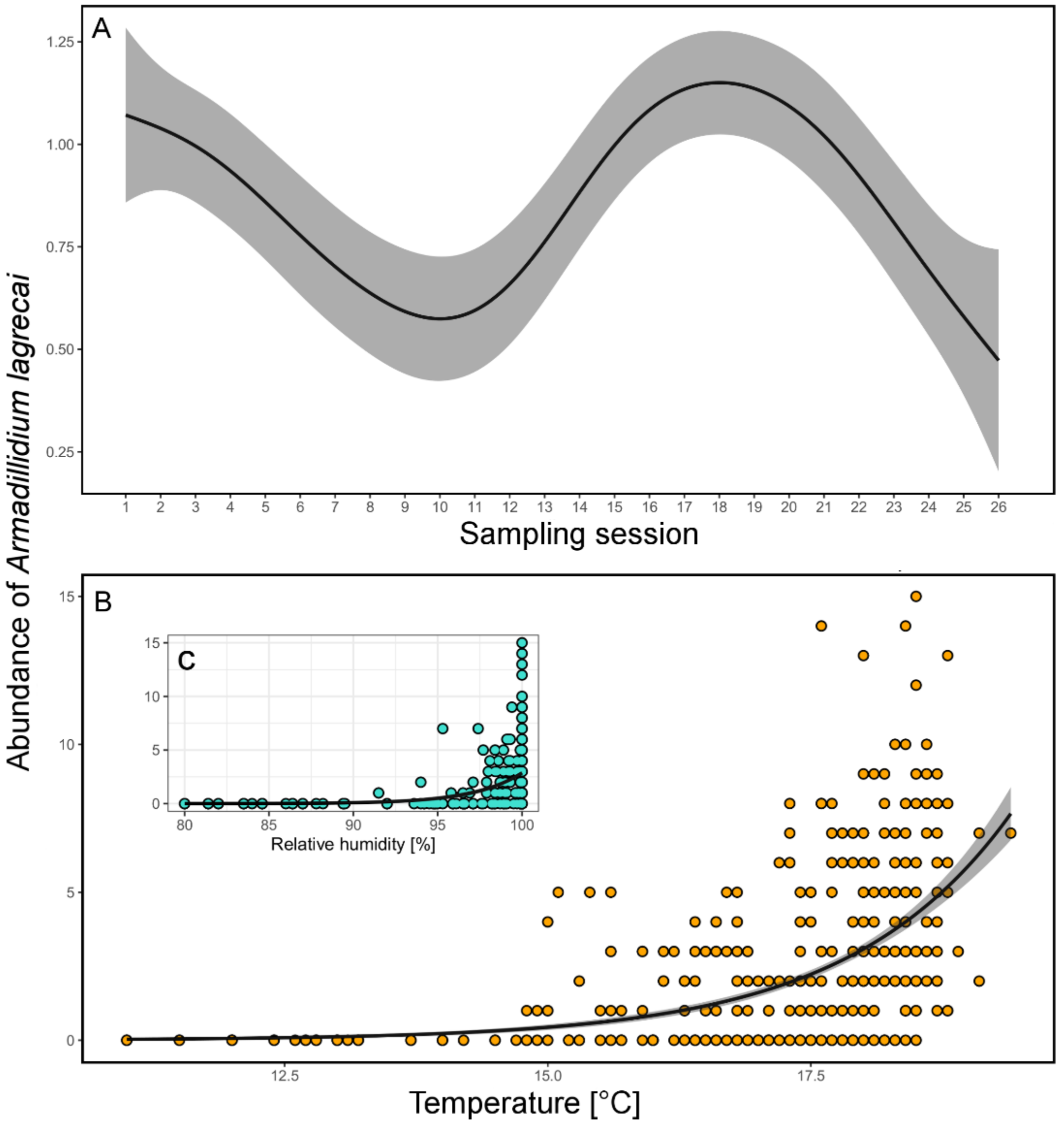

Figure 12. Abundance trends of *Armadillidium lagrecai* in the Monello Cave. Generalized additive model analysis predicted values (black line) and 95% confidence intervals (grey area) of abundance over time (smoother) (**A**); Effect of temperature on the abundance according to a generalized linear mixed model (**B**); The inset graph (**C**) shows the trend obtained using relative humidity instead of temperature as explanatory variables in the model.

None of the other factors had a significant influence on abundance, including the number of visitors and the distance from the tourist path, in accordance with previous studies (e.g., Faille et al., 2014; Pacheco et al., 2020). In other words, with a moderate regime of 68 visitors per month, the current management of the Monello Cave does not seem to harm this subterranean species. The number of visitors present during the sampling activities, as well as the rigorous path delimitation, appears to minimize disturbance.

4.3. Taxonomic characterization of microbial communities on Etna lava caves (Paper III)

In Paper III, the investigation was carried out at the ecosystem scale by providing a first preliminary insight into the microbial diversity of the Etna volcano lava tubes.

Field emission scanning electron microscopy (FESEM) documented an abundant presence of microbial cells with different morphotypes including rod-shaped, filamentous and coccoidal cells with surface appendages, resembling actinobacteria reported in other lava tubes across the world (e.g., Riquelme et al., 2015; Porca et al., 2012; Axenov-Gribanov et al., 2016).

We identified a total of 6,310 unique ASVs for the 12 samples, with the samples collected in “Grotta Lunga” being richer than the others (GL_3 = 1190 ASVs; GL_1 = 1057 ASVs). These samples also showed the highest values in terms of alpha diversity indices, i.e. Shannon index (Miller et al., 2012) and Pielou’s evenness (Shannon & Weaver, 1949) (Table 2), calculated on the ASVs.

Table 5. Statistics. Diversity indexes.

Cave name	Touristic use	Samples	Raw reads	Trimmed reads	Observed ASVs	Shannon	Pielou’s
Grotta Lunga	High	GL_1	113047	51502	1057	8.117803	0.8121241
		GL_3	107045	48489	1190	8.762284	0.8618003
Grotta del Santo	High	GS_1	132755	61396	621	5.546317	0.6012919
		GS_2A	105144	21755	410	5.578653	0.6440540
		GS_2B	128250	56174	286	2.527644	0.3113268
		GS_4	132437	58838	678	7.348656	0.7855029
Grotta Catanese I	Low	GC1_1A	105899	43269	677	5.077743	0.5432766
		GC1_2	124509	52204	743	4.797957	0.5055883
Grotta di Mt Corruccio	Low	GMC_1	125910	55677	639	4.144329	0.4468860
		GMC_2	108121	50496	569	6.867270	0.750339
		GMC_4	141225	66672	384	5.293697	0.6199319
		GMC_5	131544	56512	405	3.209504	0.3722437

The taxonomy measured at the order level showed a higher number of orders for the “Grotta Lunga” (174 orders), followed by “Grotta Catanese I” (150 orders), and then by “Grotta del Santo” and “Grotta di Monte Corruccio” caves with 148 and 147 orders respectively.

The relative abundances of the dominant phyla in each cave sample showed that most of the identified bacteria belonged to Actinomycetota, followed by Pseudomonadota, Acidobacteriota, Chloroflexota, Cyanobacteria, and other phyla with lower representativeness (Fig. 4).

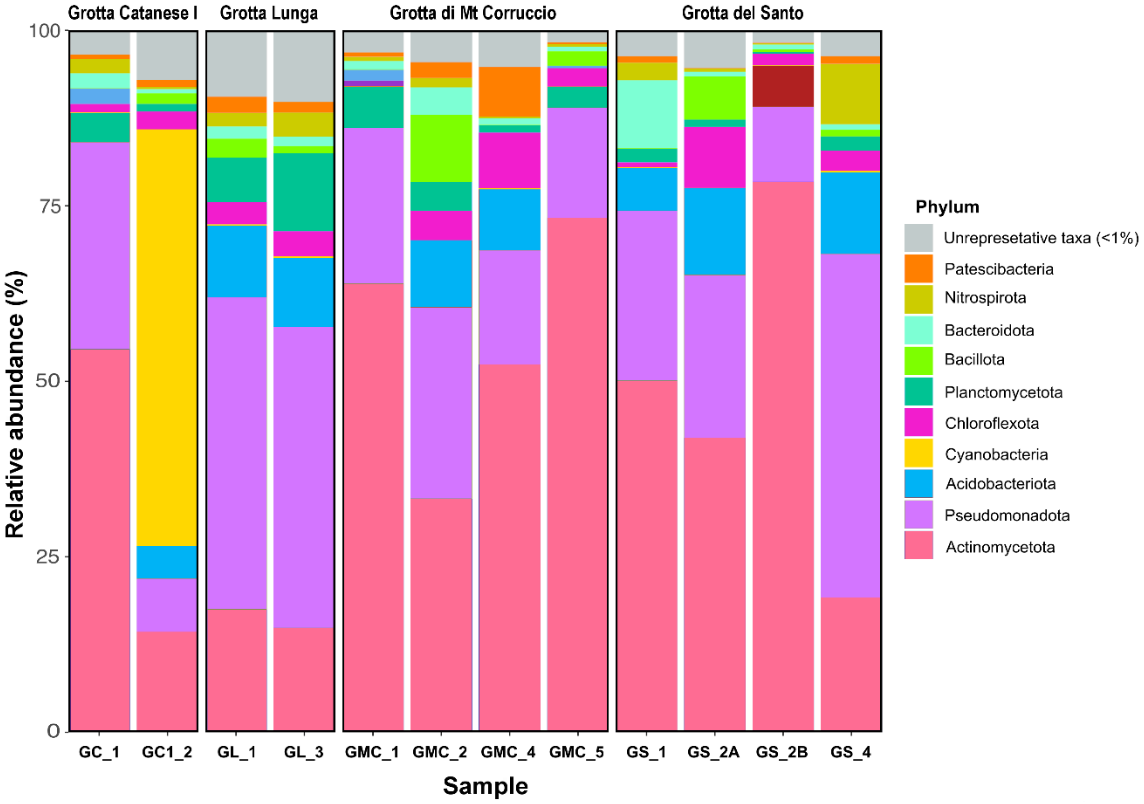

Figure 13. Distribution patterns of bacterial phyla in the samples (99% ASV cutoff).

Actinomycetes was the most abundant class in “Grotta del Santo” (GS_2B: 78.21%), and “Grotta di Monte Corruccio” (GMC_5: 72.76%; GMC_1: 62.94%) sample, while Gammaproteobacteria was predominant in “Grotta Lunga” (GL_1: 28.60%) and “Grotta del Santo” (GS_4: 25.99%). The class Gammaproteobacteria is among the most dominant group in habitats with either natural or anthropogenic organic inputs (Buresova et al., 2022). Accordingly, it seems to represent a good bioindicator to detect the presence of contaminants in soil (Jones et al., 2015; Lors et al., 2010; 2012) or caves (Niepceron et al., 2013). This taxon was abundant in samples GL_1 (28.69%), GL_3 (24.65%) from “Grotta Lunga” and GS_4 (25.99) from “Grotta del Santo”, which represent “high tourist use” caves.

At the genus level, the analysis revealed a dominance in the samples of the genus *Crossiella*, which is actively involved in biomineralization processes, followed by *Pseudomonas*, *Bacillus*, *Chujaibacter* and *Sphingomonas*. The presence of these taxa is associated with the carbon, nitrogen and ammonia cycles, and some are possibly related to the anthropic disturbance of these caves.

4.4. Urbanization, agricultural, and industrial activities: Paper IV

In Paper IV, the diversity of the invertebrate community thriving in the gypsum karst system of the Monte Conca sinkhole (Sicily, Italy) was investigated both in the sulfidic and non-sulfidic habitats. Moreover, the trophic interactions on biological assemblage were investigated by mean of stable isotope analysis.

The faunal investigation conducted by visual censuses and hand sampling methods led to the discovery of a structured biological assemblage of 54 species composed of both subterranean specialized and non-specialized species, encompassing all trophic levels.

The general epigean origin of the assemblage is related to passive water transportation during the wet season, contributing to an overall and seasonal increase of the species diversity at the inner parts of the cave. During the dry season, the lack of surface water flowing in the cave reduces the intake of epigean species, drastically reducing the overall number of species in the cave, but favoring the resident cave fauna, especially those organisms dwelling in the sulfidic habitat.

The results suggest that the existence of an autochthonous food source sustains a high biodiversity in the cave, especially in the sulfidic ponds. In particular, the community in sulfidic habitat show values isotopically light of carbon and nitrogen, indicating a chemoautotrophic food source (Fig. 5).

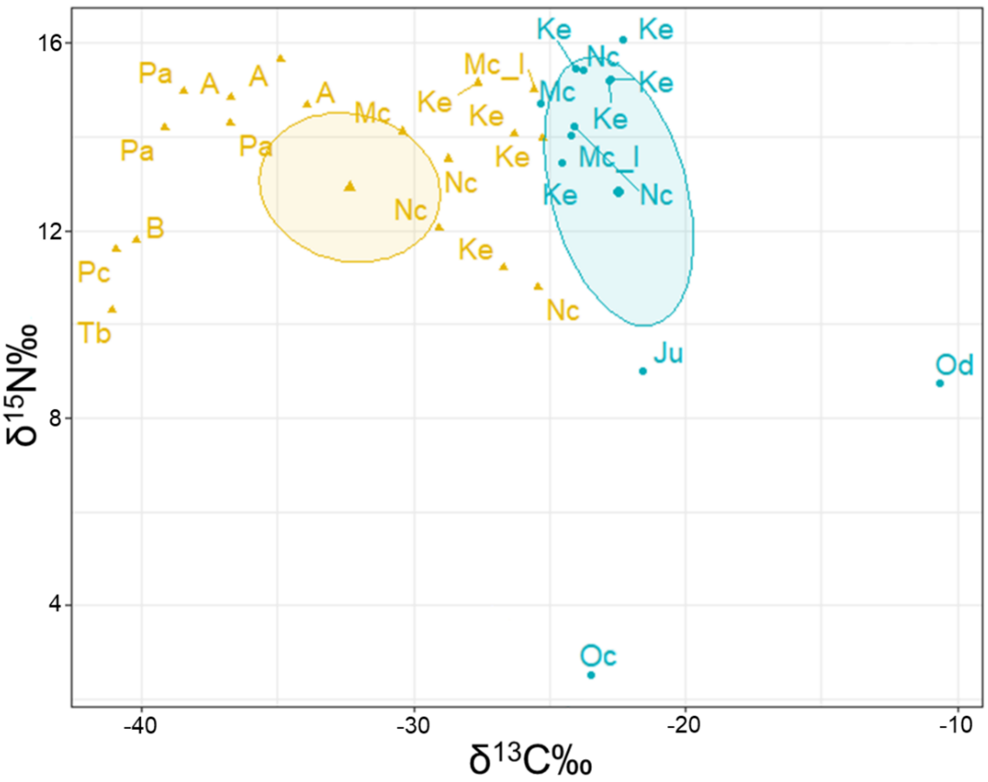

Figure 14. Scatter plot of $\delta^{15}N$ against $\delta^{13}C$ in sulfidic (yellow) and non-sulfidic (blue) habitat. The samples differ in their $\delta^{13}C$, indicating differential use of carbon sources. Abbreviations: A = *Agabus* sp., B = biofilm, Oc = organic matter collected in the control pool (non-sulfidic habitat), Ju = Julidae, Ke = *Kryptonesticus eremita*, Nc = *Nepa cinerea*, Mc = *Melademа coriacea*, Mc_l = *Meladema coriacea* (larvae), Od = *Oxychilus lagrecai*, Pa = *Paranchus albipes*, Pm = *Proasellus montalentii*, Tb = *Tubifex blanchardi*.

However, besides the driest period, the flooding during the wet season prevents the establishment of trophic chains exclusively based on chemoautotrophic production. The wet season causes large volumes of water to runoff from the surface and enter the Monte Conca sinkhole. This input facilitates the contact between the sulfidic and non-sulfidic habitats and a strong dilution of the hydrogen sulfide content of the water.

Recent observations have highlighted a constant input of water even during the summer period, probably derived from the irrigation system upstream of the cave entrance, which according to Davis et al. (2020) facilitate the entrance of microorganisms derived from anthropogenic inputs rather than sulfur oxidizing organisms, including the bacteria *Escherichia* and *Lysinibacillus*, likely derived from the agricultural regions around the Monte Conca sinkhole.

4.5. Global climate change: Paper V, VI

Paper V pointed out for the first time the presence of *Meta menardi* in Sicily, where the species is distributed along the slopes of Mount Etna in caves above 1,200 m a.s.l. reaching the maximum altitude of 1,600 m, confirming its higher frequency for colder and wettest sites seen in Mammola & Isaia, 2014. On the contrary, *Meta bourneti* prefers warmer sites, with records spanning from the coastline up to 1,200 m. Neither of the species have been observed over 1,600 meters. Considering the occurrence of both species in caves located up to 2,000 meters in other localities, this trend for *Meta* spiders on Mount Etna is likely ascribed to the unsuitable habitat in the high volcanic areas of Mount Etna (i.e. presence of lava substrates devoid of vegetation and frequently affected by volcanic activities), especially for the survival of the epigean juvenile stages (see details on life cycle in Mammola & Isaia, 2014).

Considering the high dispersal ability of both species, in a global warming scenario the two species of *Meta* would easily come into contact, especially in light of the small range of distribution of *M. menardi* on Mount Etna. Moreover that the two species have never been observed to coexist within the same hypogean site (Mammola & Isaia, 2017).

Paper VI provides a detailed analysis of the main factors contributing to segregation of the two species, in order to model their potential distribution under current and future climatic conditions at regional scale in view of the conservation of the Sicilian populations of *M. menardi*.

The PCA based on microclimatic variables has suggested a clear segregation between *M. bourneti* and *M. menardi* (Fig. 6). Temperature related variables (BIO08) seem to favour the occurrence of *M. bourneti*, but the species seems negatively influenced by precipitation-related variables (BIO19), indicating a preference of the species for warmer and drier sites. On the other side, *M. menardi* colonises sites at higher elevation where temperature is lower (slight contribution of BIO07) and the amount of precipitation is generally higher (notable contribution of BIO18).

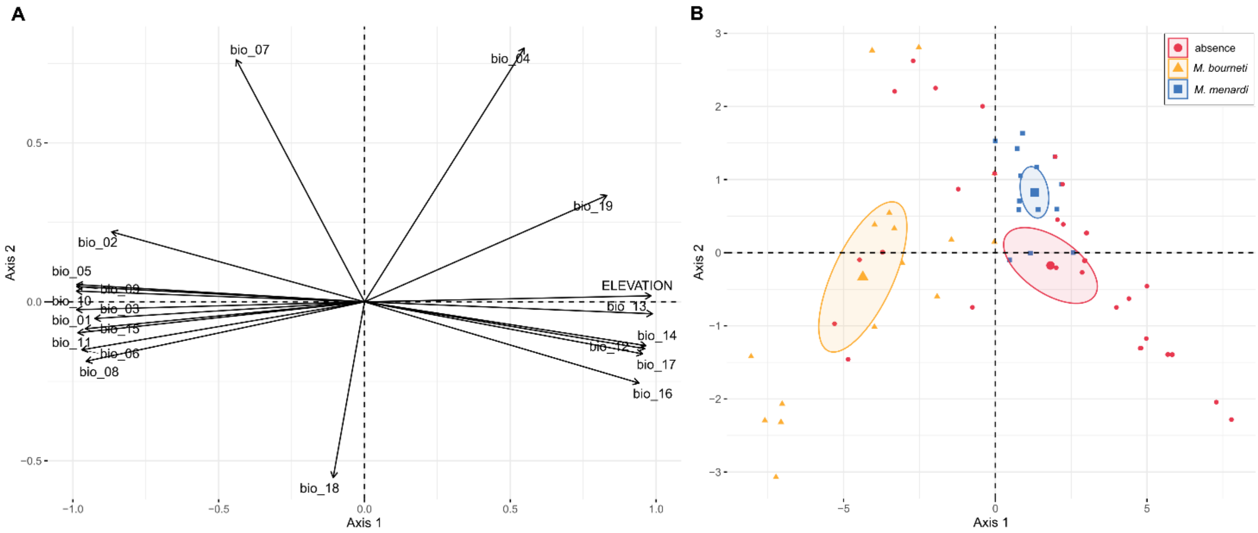

Figure 15. Results of the PCA performed on the bioclimatic variables and elevation with the arrows representing the loadings of the variables to the first two axes (**A**) and points representing the scores of each sampling site (**B**). Ellipses represent standard deviations around the centroids of the three groups of sampling sites (red = absence sites; orange = presence of *Meta bourneti*; light blue = presence of *M. menardi*).

The analysis of the niche hypervolume of the two species on Mount Etna has shown a marked difference of their four-dimensional hypervolume (Fig. 7). *M. menardi* showed a markable smaller bioclimatic niche compared to *M. bourneti*. Moreover, the niche hypervolume of *M. menardi* results almost completely comprised within that of *M. bourneti*. This suggests the ability of both species to exploit a similar variety of microhabitats in subterranean habitat as highlighted in other study areas (Mammola & Isaia, 2014; Mammola & Isaia, 2017; Nentwig et al., 2017; Smithers, 2005), although *M. bourneti* seems to exhibit a higher ecological plasticity in terms of microclimatic requirements.

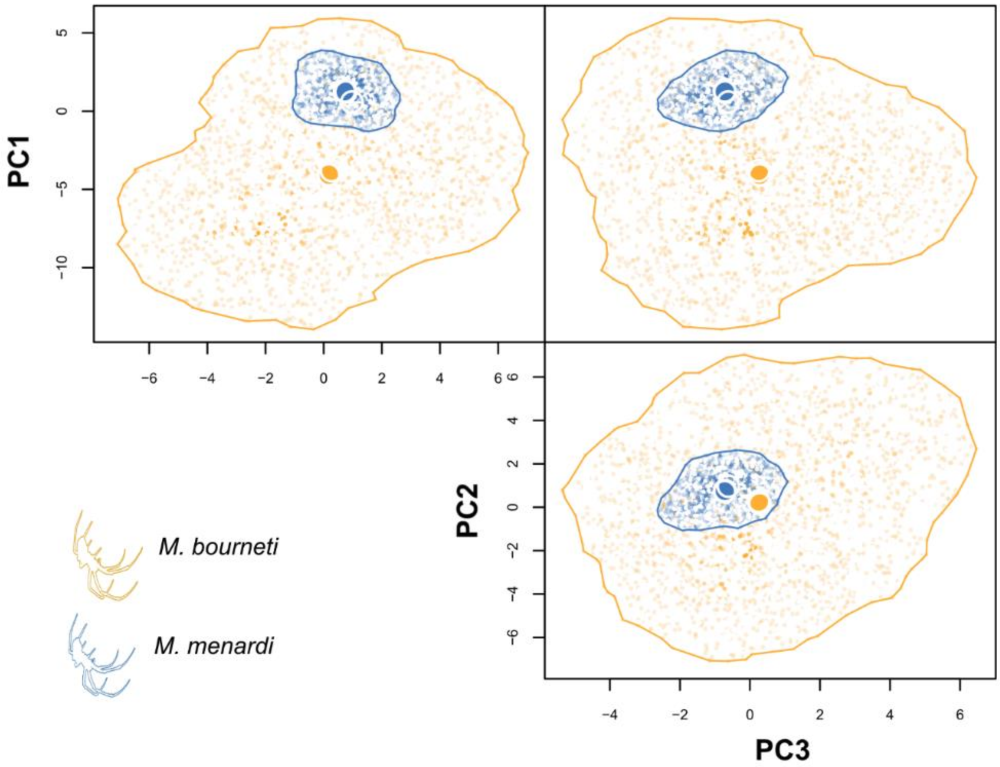

Figure 16. Pair plots showing the estimated three-dimensional hypervolumes for the two *Meta* spider (orange = *M. bourneti*; light blue = *M. menardi*). The colored points for each taxon reflect the centroids (large points), original observations (intermediate points) and the stochastic points sampled from the inferred hypervolume (small points).

Neither of the species seems able to colonise volcanic caves of Mount Etna over 1,600 meters, probably due to unfavorable microclimatic conditions or, more likely, to the lack of suitable habitat. These mainly represent areas of recent lava fields devoid of vegetation due to the major frequency of volcanic events (i.e., volcanic ash fall and presence of lava flows).

The suitable areas predicted by the models for both species agreed with its current range distribution on Mount Etna (Fig. 6A, B). For *M. bourneti*, the current suitable habitats were predicted along all the slopes of the volcano, from the sea level up to 1,100 meters, from where *M. menardi* starts to be found. For *M. menardi*, the present distribution was predicted along a narrow strip on Mount Etna, between the areas colonised by *M. bourneti*, and those at higher altitudes interested by recent volcanic activity.

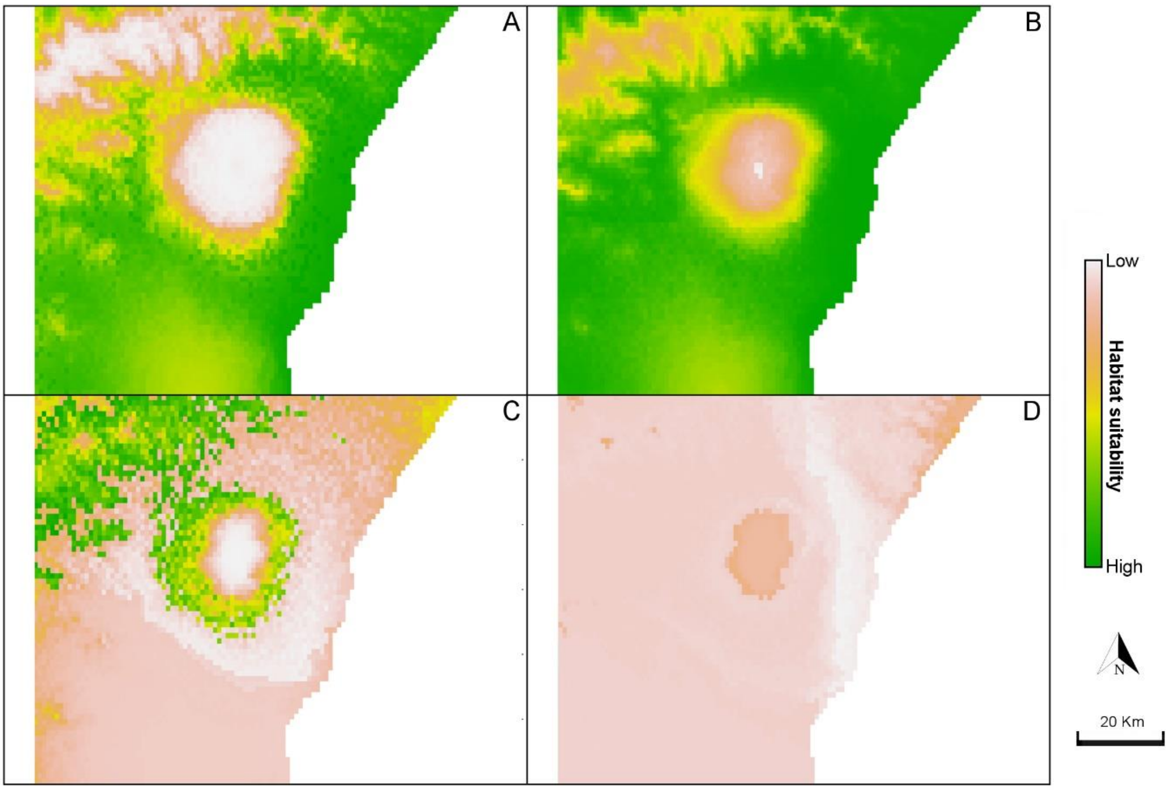

Figure 17. Predicted current (1970-2000) and future (2070) under the high-emission scenario RCP 8.5 distribution for *Meta bourneti* (A and B, respectively) and for *M. menardi* on Mount Etna (C and D, respectively). All predictions are based on ensemble of GLM, BRT and FDA models.

The averaged future predictions from the three climatic models for 2070 under RCP 8.5 scenario, showed that both species will be affected by climate change (Fig. 6C, D). *M. bourneti* is likely to be favoured by the increase of temperature and will expand its distribution toward new suitable areas up to 1,300 meters possibly colonizing in caves currently occupied by its counterpart *M. menardi*. At the same time, predictions for *M. menardi* would potentially lead to the abandonment of southern caves on Mount Etna, causing a significant loss of suitable areas, likely determining the disappearance of the species from the study area due to the global change (Fig. 6D).

4.6. The presence of non-native species introductions: Paper VII

In Paper VII, the available knowledge on alien species in subterranean ecosystems globally were summaries. We included 104 publications in the final database, encompassing 362 reports of alien species in subterranean habitats corresponding to 246 unique alien species invading subterranean habitats. Most of the subterranean alien species were reported in terrestrial subterranean habitats (322 out of 362 cases; 88.9%), rather than freshwater (40; 11.0%). Subterranean lithic was the most invaded terrestrial ecosystem (323 cases) of which 194 (53.6 %) in limestone caves and 129 (35.6%) in lava caves, followed by anthropogenic subterranean voids with 14 cases (3.87%).

Most detected alien species are mostly invertebrates (211; 85.8%), followed by vertebrates (20; 8.1%) and plants (15; 6.1%) (Fig. 9A). Among invertebrates, arthropods highly dominated the search, especially the class Insecta (59 species; 24.0%) followed by Arachnida (46; 18.7%), Entognatha (32; 13.0%), and Diplopoda (19; 7.7%) (Fig. 9A).

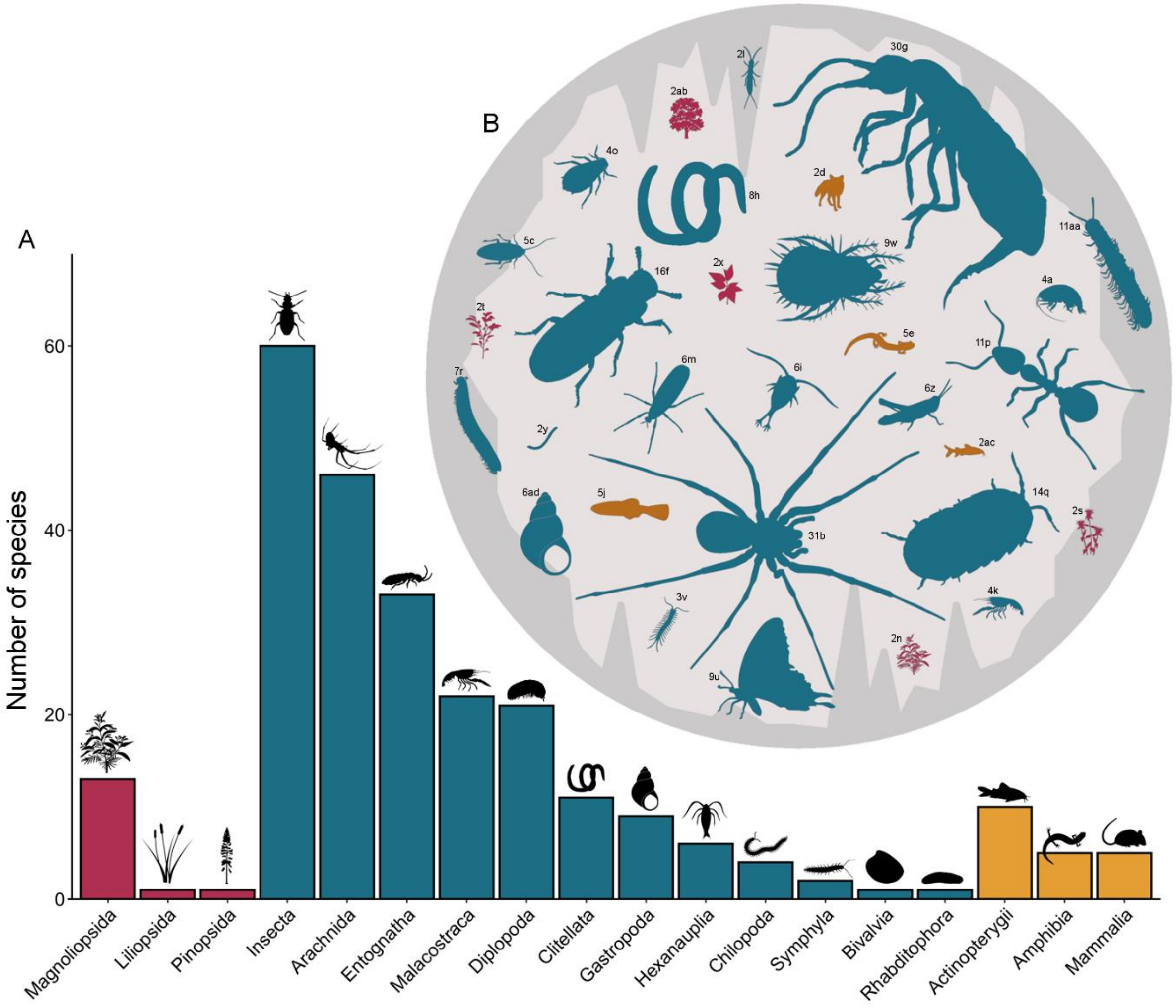

Figure 18. Barplot representing the number of alien subterranean species within each taxonomic class (total number of species: N = 246) (**A**); Number of alien subterranean species within each taxonomic order (orders with less than two species are not included) (**B**). The silhouette size for each order is proportional to the number of species detected through the literature survey. Each order is represented by an illustrative example. a: Amphipoda; b: Araneae; c: Blattodea; d: Carnivora; e: Caudata; f: Coleoptera; g: Collembola; h: Crassiclitellata; i: Cyclopoida; j: Cyprinodontiformes; k: Decapoda; l: Diplura; m: Diptera; n: Fabales; o: Hemiptera; p: Hymenoptera; q: Isopoda; r: Julida; s: Lamiales; t: Laurales; u: Lepidoptera; v: Lithobiomorpha; w: Mesostigmata; x: Myrtales; y: Opisthopora; z: Orthoptera; aa: Polydesmida; ab: Sapindales; ac: Siluriformes; ad: Stylommatophora.

Araneae and Collembola were the most dominant orders, represented respectively by 31 species (12.6%) and 30 species (12.2%), followed by Coleoptera (16; 6.5%), Isopoda (14; 5.7%), and Hymenoptera (10; 4.1%) (Fig. 9B).

Palearctic (116; 47.2%) and Nearctic (18; 7.3%) biogeographic regions represented the main source of alien species, with broad bi-directional exchanges between these two regions (Fig. 4). This trend is likely ascribed to the greater economic development of these regions and their associated international trade and globalisation (Turbelin et al., 2017), although could be also attributed to the higher research effort on alien species (Pyšek et al., 2008).

Information on pathways of biological invasions in subterranean species is scarce. We could retrieve information on the routes by which alien species were introduced into the recipient country for only 64 out of 362 cases (17.7%). Of these, the most widespread form of introduction is related to trade activities (54 out of 64 cases, 84.3%: Transport – contaminant, 31 cases, 48.4%; Transport – stowaway, 36 species, 35.9%).

Information on the mechanisms through which alien species impact native subterranean organisms and/or ecosystems was available for 67 cases, with the most important being competition (40/67 cases; 59.7%) and predation (26; 38.8%), followed by disease transmission (7; 10.4%) and parasitism (4; 6.0%).

Information on costs associated with alien species in subterranean ecosystems is very limited. This may not come as a surprise as many of these species are invertebrates, which are generally underrepresented in the literature (Cardoso et al., 2011; Titley et al., 2017).

Only in a limited number of cases (22 out of 362) have alien species been managed in subterranean ecosystems. Furthermore, the effectiveness of these interventions has seldom been tested statistically (Mammola et al., 2022), and most knowledge on eradication activities remains qualitative (Simberloff, 2002; Genovesi, 2005).

Of the 246 alien species listed in the dataset, 51.6% is considered to be successfully naturalised in subterranean habitats. Only some of them exhibit one or more adaptations to subterranean life (90; 36.6%). Moreover, the number of alien species strictly adapted to subterranean environments is very limited, probably due to the high sensitivity of these species, even the smallest environmental variations (e.g., Barr & Kuehne, 1971; Howarth, 1980; Culver, 2005) limiting their dispersion outside. According to our prediction, the presence of adaptive traits is the strongest predictor of the probability of a species to become established in a subterranean habitat. No other traits exert a strong effect on the probability of becoming established in a subterranean habitat (Tab. 3).

Table 6. Estimated regression parameters according to a Bernoulli generalised linear mixed model (GLMM) investigating the drivers of established alien species in subterranean habitats. For predictor variables, we report in square brackets the level that is being tested.

Predictor	Estimate	S.E.	*z*	*P*
Intercept	–0.693	0.480	–1.445	0.148
Adaptive trait [Yes]	1.436	0.378	3.803	**<0.001**
Presence of wings [Yes]	0.427	0.544	0.784	0.432
Trophic level [Others]	–1.078	–0.662	–1.628	0.104
Trophic level [Omnivores]	0.263	0.505	0.522	0.602
Trophic level [Predators]	0.329	0.558	0.590	0.556

5. Conclusion and perspectives

Subterranean ecosystems are some of the most understudied ecosystems on Earth and containing a high diversity of specialised organisms showing peculiar morphological, behavioral, and physiological adaptations (Culver & Pipan, 2019; Romero, 2009).

These environments and their biota are still superficially known. Most obligate subterranean species worldwide have in fact not yet been discovered and may face extinction before they are discovered and formally described (Zagmajster et al., 2018). Moreover, subterranean habitats are among the most threatened and vulnerable in the world, due to their fragility and relatively low resilience (Trajano, 1992; Culver & Pipan, 2019; Elliott, 2000; Hamilton-Smith & Eberhard, 2000).

In light of the recent decline in biodiversity (Butchart et al., 2010), it is urgent to make resolute decisions for the conservation of the subterranean realm and the organism living therein.

A scientifically informed protection of subterranean realm and its biodiversity is thus a matter of the highest importance (Mammola et al., 2019a). Identifying the main stressors and their effects on subterranean habitats is in fact key to proposing actions to protect and sustainably conserve these ecosystems at local or wider scale (i.e., globally). Despite the growing literature concerning the subterranean biome and the extent of potential threats, some threats result still largely unexplored, especially those devoted to understanding climate change effects, alien species, pathogens, and overexploitation (Mammola et al., 2022).

To this end, this thesis dealed with some of the most widespread threats in subterranean habitats, focusing on both emerging global threats such as climate change and still unexplored threats as the invasion of alien species in subterranean habitats, also providing practical suggestions and future directions for management practices.

The threat “recreational use of caves” has been addressed in Papers I, II, III across different taxa at ecosystem scale. Paper I examined the threat of the growth of photosynthetic organisms in caves opened for tourist activities (i.e. Show Cave). Despite this threat being extensively covered in literature (e.g. Mulec & Kosi, 2009; Baquedano et al., 2019; Mulec, 2019), Paper I develop one of the few case studies on the effective contribution of reducing the lampenflora by the modifications of the light regime (but see Borderie et al., 2014). We also provided suggestions to implement appropriate management actions addressed to the modulation of the lighting regime in show caves, especially in the areas most exposed to the proliferation of lampenflora. According to our results, the modulation of the lighting regime can help in limiting the growth of photosynthetic biofilms in show caves. Thus, the adoption of certain behaviors such as the installation of low-intensity lights or the use of only portable lights can prevent or, in any case, reduce the proliferation of lampenflora in caves. Future direction should therefore foster the development of recovery plans with cave managers which can also be applied on a regional or national scale, in order to ensure the creation of management plans that can favor effective conservation of these environments.

Accordingly, effective and informed management, including a scheduled visitor regime that takes into account the cave size or the presence of vulnerable species can also reduce the impact on subterranean biodiversity, a point that was highlighted in Paper II. The study investigated the factors affecting the presence and distribution of the subterranean isopod *Armadillidium lagrecai*. Our results have shown a preference for those cave sectors where the microclimate is mostly constant during the year. The observed abundance fluctuations were determined by the microclimatic regime that occurs naturally inside the cave rather than by any disturbances caused by visitors, contrary to other investigations where tourist seems to negatively affect the presence of invertebrates (Isaia et al., 2011; Pellegrini & Ferreira, 2012, 2016; Alonso et al., 2019).

Under the current management, it appears that the existing visitor's regime in the Monello Cave has no effect on the abundance and local distribution of *A. lagrecai*. However, it cannot be excluded that a greater number of visitors could alter the cave microclimate in terms of temperature and humidity (Baker & Genty, 1998; Dominguez-Villar et al., 2010). Thus, the presence of visitors should continue to be accurately monitored with microclimatic surveys and visitor limitations maintained as suggested to the manager of the Monello cave.

Despite microbes and viruses play a critical role in ecosystem functioning including subterraena ones (Griebler & Lueders, 2009; Griebler et al., 2014), yet, limited studies have been focused on understanding the role of pathogens in subterranean ecosystems (Mammola et al., 2022; but see Jurado et al., 2010). In Paper III, we provide a first insight into the microbial communities of Mount Etna lava tubes. Although this represents a preliminary investigation based on a limited number of samples, our results revealed a great diversity and abundant presence of taxon, among which several phylotypes previously detected in other lava tubes worldwide. The differences in terms of richness detected among caves could be ascribed to the different environmental conditions as well as a possible response of the microbial communities to human disturbance (Mammola et al., 2017). Further investigations will be therefore necessary to ascertain the presence of contaminants and their potential risk both to the natural environment and human health represented by opportunistic pathogens.

The threat "urbanization, agricultural, and industrial activities" has been addressed in Paper IV at ecosystem scale in Monte Conca sinkhole. The investigation has detected a rich invertebrate community within the cave that relies seasonally on autochthonous food produced chemoautotrophically by sulfur-oxidizing bacteria. Despite being included within a Strict Nature Reserve, this cave is highly threatened by the presence of agricultural activities that pose in danger to this fragile ecosystem by both the input of contaminants of anthropogenic origin and the alteration of the natural hydrological regime of the sinkhole. Such alterations could lead to severe disturbance of this exceptional ecosystem, thus threatening its existence. Cave protection is therefore ineffective if anthropic impacts, such as agricultural activities, alter the land use overlying the cave or the water regime nearby (e.g. Davis et al., 2021; Marmonier et al., 2013; Di Lorenzo et al., 2014). Due to its considerable scientific importance, Monte Conca sinkhole needs immediate and unquestionable protection.

The threat global climate change has been investigated regionally in Papers V and VI.

In recent years, there is a growing concern over the threat of global climate change. More appropriate management plans need to be established in light of a possible rise in temperature, a situation that poses in dangers both terrestrial and aquatic subterranean ecosystems, and the species living therein whose distribution is limited by their strict environmental requirements (Pipan et al., 2018; Taylor et al., 2013).

The well-defined microclimatic range makes the genus *Meta* an interesting target of research and a suitable model for the study of the effect of climatic changes at a local or global scale. It seems likely that the replacement of *M. menardi* by *Meta bourneti*, followed by an upward expansion of the latter will occur in the short term. The potential expansion of *M. menardi* in the summit of the study area will be limited by the environmental barrier represented by the young lava substrate, which represent a hostile environment for the epigean juvenile phase of *M. menardi* (Paper V). It is in fact unlikely that the vegetation, whose presence is crucial for the dispersion of the species, will shift its distribution in the upper section of the volcano frequently affected by the volcano activities, due to the rather long colonisation times of the vegetation (Poli & Grillo, 2000).

Considering that local populations of many species cannot shift their climatic niches rapidly enough to prevent extinction (Román-Palacios & Wiens, 2020), and the difficulty of planning prevention strategies to reduce impacts of global climate change on a national and global scale (Prabhakar et al., 2009), investigations and monitoring activities at the local level would be necessary to establish an appropriate management plan for the conservation of the population of *M. menardi* in Sicily.

Lastly, the threat “non-native species introductions” in subterranean ecosystems has been investigated at the global scale in Paper IV. The study represents the first contribution on this topic, which aims to shed light on broader questions about taxonomic biases, geographical patterns, modes of dispersal, pathways for introductions, and potential impacts. It underlined several gaps in the knowledge and research on this topic, in contrast with the available information on alien species colonizing surface habitats. Our data suggested that only a limited number of alien species may represent a real threat to subterranean ecosystems and to the species living therein, probably due to the strong ecological filter imposed by subterranean systems (Culver & Pipan, 2019). However, greater awareness and research efforts on the presence and distribution of alien species would be crucial to effectively prevent and manage their expansion (CBD, 2000). Accordingly, researchers in the field of subterranean biology should take into account the presence of alien species when preparing species inventory besides considering the presence of endemism and rareness. Greater awareness of the presence and distribution of alien species would fill the gap in the potential distribution and spread of alien invertebrate species in subterranean habitats, laying the foundations for future management practices and interventions. Indeed, no management practices can be recommended until the relationships between native and alien species have been well documented (Reeves, 1999).

6. Acknowledgments

First and foremost, I am extremely grateful to my supervisor Marco Isaia for providing me with this great opportunity. Without his invaluable guidance, enthusiastic encouragement and effective critiques this PhD would not have been achievable.

A special thanks go to Elena Piano for her advice and help during my PhD path, especially for giving me valuable insights into statistics (and be always available for morning coffee!).

Thanks, should also go to Filippo Milano with whom I partly shared this "journey" and who helped me in numerous ways during various stages of my PhD.

Thank also to Stefano Mammola for his valuable help and contribution on Papers II and VII.

I am also very grateful to Ana Miller, for warmly welcoming me into her lab and letting me join the sampling campaign in La Palmas Island.

I would like to thank all team members from the Centro Speleologico Etneo (CSE), and especially Francesco Leone whose constant and valuable support made the field activities possible on Mount Etna lava caves.

Finally, I would like to express my gratitude to my family and friend. Without their support and encouragement over the past few years, it would be impossible for me to complete my path.

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Paper I

Article published in *Journal for Nature Conservation* 64 (2021): 126075.

Modulating lighting regime favours a sustainable use of show caves: a case study in NW-Italy

Elena Piano*[1], Giuseppe Nicolosi[1], Marco Isaia[1]

[1]Laboratory of Terrestrial Ecosystems, Department of Life Sciences and Systems Biology, University of Turin, Via Accademia Albertina 13, 10123 Torino, Italy

*corresponding author: elena.piano@unito.it

ABSTRACT

When artificial lighting systems are installed in show caves, alien photosynthetic epilithic prokaryotic and eukaryotic microorganisms —the so-called 'lampenflora'— form extended biofilms on cave speleothems, causing structural and aesthetic damage. Among the environmental drivers influencing their proliferation, light intensity and duration of illumination are expected to play a key role. To unravel which environmental variables determine the proliferation of lampenflora, we designed a specific study in two Italian show caves with similar characteristics and management practices, but differing in terms of tourist use and thus in terms of duration of illumination. In each cave, we selected multiple illuminated speleothems at progressive distance from the cave entrance where we measured the chlorophyll-a concentration of the three main microorganism groups constituting phototrophic biofilms, namely cyanobacteria, diatoms and green algae, by means of the portable fluorimeter BenthoTorch®. In each sampling plot we also recorded the intensity of artificial lights and we visually evaluated the presence of seeping water on speleothems. By adopting advanced regression techniques, we revealed that the total chlorophyll-a and the concentration of the three groups differ between caves, increasing in the cave with higher number of visitors. Regarding the other environmental factors, light intensity positively influenced the total chlorophyll-a and the concentration of cyanobacteria, while humidity increased diatom concentration. We then provided suggestions to implement appropriate management actions addressed to the modulation of the lighting regime in show caves, especially in the areas most exposed to the proliferation of lampenflora.

KEYWORDS: lampenflora, tourist caves, BenthoTorch®, phototrophic biofilms, GLMM, chlorophyll-a concentration

1. INTRODUCTION

Caves are unique landscape features, especially in karst regions, valued for their exceptional archaeological, palaeontological, geological, and biological heritage worldwide. Several caves have been converted into tourist attractions, i.e. show caves, being visited by millions of visitors every year (Cigna, 2016), playing an important cultural and economic role (Parga-Dans, González & Enríquez, 2020). However, there is an inevitable conflict between concurrent goals of facilitating cave recreation for public enjoyment on the one hand, and protection of the subterranean environment on the other (Crane & Fletcher, 2016).

Caves are energy-poor ecosystems characterized by the absence of light, spatial confinement, climatic stability, and low biodiversity (Poulson & White, 1969; Mammola, 2019; Culver & Pipan, 2019). Given these peculiar conditions, these ecosystems are often highly susceptible to anthropogenic pressures (Cigna, 2016; Mammola et al., 2019). When wild caves are converted into tourist sites, the subterranean environment is severely transformed by the construction of trails, the presence of visitors and the installation of artificial lights (Cigna, 2019). These changes impose an ecological pressure upon the cave ecosystem (Pulido-Bosch, Martin-Rosales, López-Chicano, Rodriguez-Navarro, & Vallejos, 1997), with significant alterations on the abiotic (Lang, Faimon, & Ek, 2015a, b; Šebela, Prelovšek, & Turk, 2013; Šebela, Baker, & Luke, 2019) and biotic components (Isaia, Giachino, Sapino, Casale & Badino, 2011; Lim, Cappelle, Hoem, & Furey, 2018; Mann, Steidl, & Dalton, 2002; Nicolosi et al., 2021). Visitors alter the local microclimate by increasing temperature and CO_2 concentrations (Cuevas-González et al., 2010; Novas et al., 2017) and spread around lint, fungal spores and bacteria from the outside (Chelius et al., 2009; Mammola et al., 2017; Saiz-Jimenez, 2012; Zhelyazkova et al., 2020), while the installation of artificial lighting systems favours the growth of photosynthetic organisms —usually limited to the twilight zone, where light radiation is available (Nikolić et al., 2020)— on cave walls and speleothems even in the deepest parts of the cave, i.e. the so-called 'lampenflora' (Baquedano Estevez, Moreno Merino, de la Losa Román & Duran Valsero, 2019, Cigna, 2011, Falasco et al., 2014; Mulec, Kosi & Vrhovšek, 2008; Mulec, 2019; Piano et al., 2015).

Lampenflora is mainly constituted by prokaryotic and eukaryotic microorganisms of various taxonomic groups, among which Cyanobacteria, diatoms (Bacillariophyta) and green algae (Chlorophyta) are the most abundant (Roldán & Hernández-Mariné, 2009). In the last successional stages, bryophytes, ferns and vascular plants may also occur (Castello, 2014; Mulec & Kubešova, 2010). The lampenflora growth represents a severe threat to the subterranean environment because of the dramatic changes they may have on the whole hypogean ecosystems (Albertano, 2012; Baquedano-Estevez et al., 2019). First, lampenflora has an aesthetic impact, forming thick green, brown, greyish patinas on cave walls, ceiling and paths (Mulec & Kosi, 2009). Second, microorganisms can produce biofilms by secreting a hydrated matrix of extracellular polymeric substances (EPS) that induce the adsorption of cations and dissolved organic molecules from the mineral surface causing the deterioration of the substrate (biocorrosion, Albertano et al., 2003). Third, some species are able to penetrate into the rock, causing physical alterations that include structural changes and disintegration of the substrate through mechanical pressure (Caneva et al., 2008; Warscheid & Braams 2000). Finally, dead masses generated by biofilms introduce a substantial amount of nutrients that may become available to consumers, likely favouring opportunistic species of bacteria and fungi (Baquedano Estevez et al., 2019). In addition, when the lampenflora, either living or dead, becomes encrusted in the calcium carbonate substrate, the amorphous mixture of dead microorganisms and carbonate irreversibly alters speleothems and other rock formations (Mulec, 2019).

Controlling the growth of photosynthetic biofilms is therefore a primary concern of show cave managers (Grobbelaar, 2000; Mulec, 2014). Quantifying the effect of the main environmental drivers that favour lampenflora proliferation to predict the most threatened areas is therefore pivotal to address effective management practices (Borderie et al., 2014; Piano et al., 2015). Similar studies were performed with reference to variations in carbon dioxide (Guirado, Ramos-López, Maldonado, Moreno-Balcázar, & Calaforra, 2019), temperature (Šebela et al., 2019) and airborne microorganisms (Porca et al., 2011). In particular, the subterranean environment is naturally dark, therefore the lack of light represents the most limiting factor for photosynthetic microorganisms (Falasco et al., 2014; Martinez & Asencio, 2010). Moreover, it is the only parameter that can be controlled *in situ* in terms of both light type and lighting regime (Albertano et al., 2000; Albertano, 2012; Hoffmann, 2002; Urzi, De Leo, Bruno, & Albertano, 2010). On the one hand, modifications to the light type may include turning halogen lighting systems into low temperature LED lights (D'Agostino, Beccarisi, Camassa & Febbroriello, 2015; Mulec, 2014; Havlena, Kieft, Veni, Horrocks & Jones, 2021), or using green lights (Roldán, Oliva, Gónzalez del Valle, Saiz-Jimenez & Hernández-Mariné, 2006), but their effective contribution to reducing the lampenflora growth is limited (but see Bruno & Valle, 2017). On the other hand, the modulation of light regime, both in terms of lighting duration and light intensity, is expected to significantly influence the proliferation of lampenflora (Cigna, 2011). Unravelling the role of these parameters is, however, far from trivial as the light regime strictly interacts with several other environmental factors (Baquedano Estevez et al., 2019). Therefore, the effect of both lighting regime, here intended as number of illumination hours and light intensity, and environmental parameters should be simultaneously tested to identify the most vulnerable areas in show caves. In addition, as photosynthetic communities within caves are rather complex, not only the overall pattern but also the differential distribution of the main groups composing lampenflora should be untangled in order to shed light on the distribution and proliferation of photosynthetic biofilms within show caves (Borderie et al., 2014; Piano et al., 2015).

To achieve this aim, we compared the chl-a concentration of cyanobacteria, diatoms and green algae in two show caves in NW-Italy (Toirano and Valdemino) sharing similar environmental conditions and management, but experiencing different tourist use, and therefore lightning duration. More specifically, we tested whether the show cave experiencing a higher number of illumination hours displays a significantly more extensive lampenflora growth and which microorganism group is mostly favoured. In addition, we also tested whether light intensity and other environmental parameters could affect the lampenflora growth within each cave and their differential effect on the three examined photosynthetic groups. We hypothesized that: i) a higher number of illumination hours would increase the overall growth of lampenflora by positively affecting the three examined photosynthetic groups; and ii) the three examined photosynthetic groups would show a differential response to the other environmental parameters. By combining the obtained results, we will be able to highlight the combination of environmental conditions enhancing the lampenflora proliferation and provide suggestions for the implementation of management practices.

2. MATERIALS AND METHODS

2.1. Study sites

The two study sites are located in Liguria region (NW-Italy) at 15 km of distance one from the other (Fig. 1a). Geologically, the area belongs to the Briançonnais domain of the Ligurian Alps and the *San Pietro dei Monti* Formation, which is mainly constituted by Middle Triassic dolomitic limestone rocks (Boni, Cerro, Gianotti & Vanossi, 1971; Cavallo, 2001). The microclimate is similar in the two caves, with temperature around 15°C (see Table S1 in Supplementary Material 1).

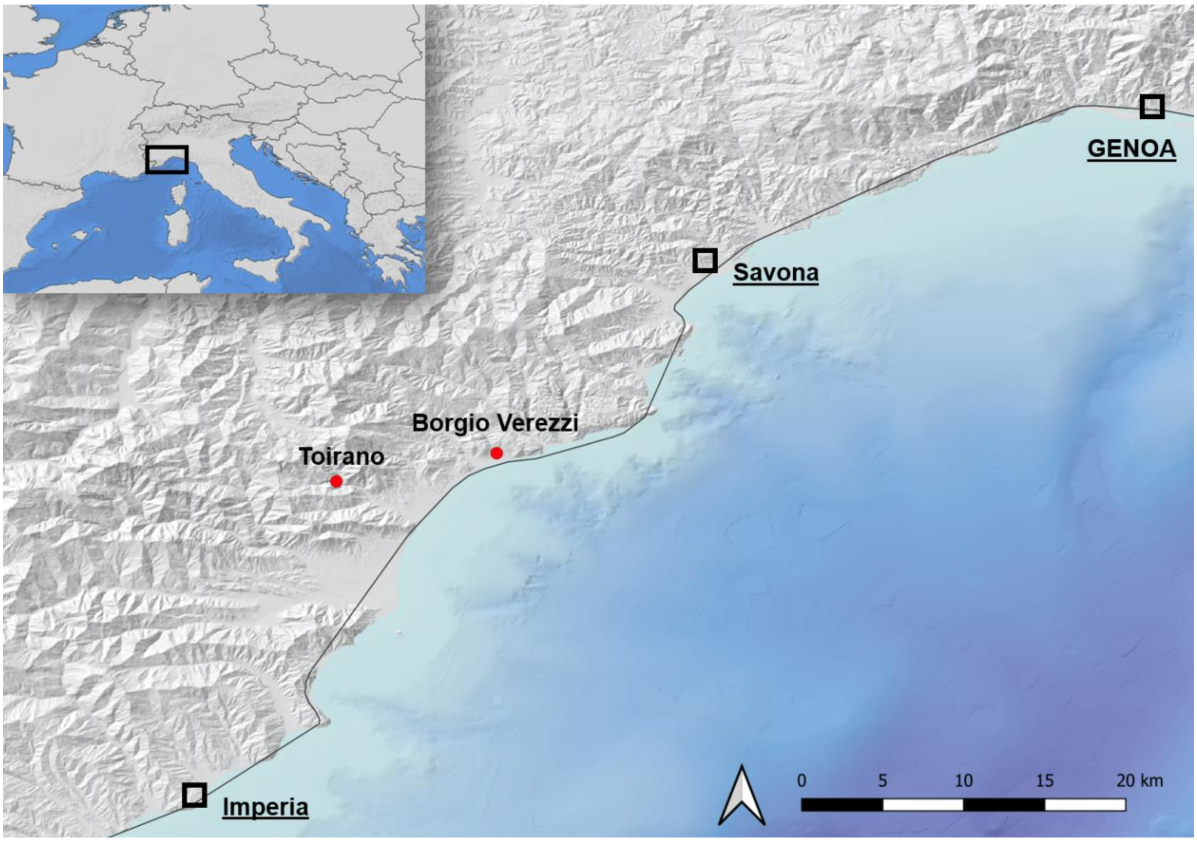

Figure 1. Map showing the geographic location of the two show caves considered in the study.

The Toirano show cave has been opened in 1953 (67 years ago) and it is visited by an average number of 75,000 visitors per year (data provided from cave managers referring to the 2019). The Toirano karst system is composed of 5 different caves, but only two of them are open to the public, namely the Grotta di Santa Lucia inferiore ([Li 59], 8.2032°E, 44.1344°N; 201 m a.s.l., 778 m long) and the Bàsura Cave ([Li 55], 8.2020°E, 44.1378°N; 186 m a.s.l., 890 m long), located about 1 km north of the town of Toirano and extending in Mount St. Pietro with an elevation gain of +20/-22 m relative to the entrance level. The morphology of the two caves suggests that they were generated by slowly flowing waters following an ascending path and subsequently shaped by condensation-corrosion phenomena due to the convection of external warm and wet air masses, and vapours produced by bats and decay of guano deposits (Columbu et al., 2021). The two caves are connected with an artificial tunnel and sealed with compartment doors. This site attracts tourists mainly because of its exceptional prehistoric and palaeontological heritage found in the Bàsura Cave, consisting of a deposit of *Ursus spelaeus* bones and traces of human activities, especially footprints, dating back to 14 cal. ky BP (Romano et al., 2019). The tourist path is 1200 m and it is equipped with a manual step-by-step illumination system, constituted by halogen lights, which are progressively turned on and off during the visits by the guides. The highest tourist flux is recorded in summer. Guided tours last more or less 60 minutes and each group is composed, on average, of 40 tourists, for a total of 1,900 tours per year. We therefore assumed that this cave is subjected, on average, to 1,900 illumination hours per year.

The Valdemino (or Borgio Verezzi) show cave ([Li 160], 8.3042°E, 44.1628°N; 30 m a.s.l., 700 m long) has been discovered in 1933 and subsequently opened to tourists in 1970 (50 years ago). It is visited by an average number of 30,000 visitors per year and the highest tourist flux is recorded in summer (data provided from cave managers referring to the 2019). The cave has been carved by the Battorezza river —which infiltrates underground during floods and whose water reaches the subterranean lake located at the bottom of the cave— and by subsequent movements of rock formations. The cave is rich in palaeontological remains dated between 750,000 and 500,000 years ago (e.g. Delfino, 2004; Ghezzo, Boscaini, Madurell-Malapeira & Rook, 2015). The tourist entrance opens at 35 m a.s.l. and it is sealed with a compartment door, while a second entrance opens few meters above. The cave has a total planimetric development of 700 m and a descending trend, with the lowest part of the cave that reaches the sea level. As a consequence, in some lakes in the deepest section, seawater mixes with freshwater. The tourist path is 800 m long, with a manual step-by-step illumination system that, at the time of our sampling, was composed by halogen and incandescent lights progressively turned on and off during the visits by the guides (for the record, the illumination system was replaced with LED lights in 2021, after this research took place). Incandescent lights were placed at < 1 m of distance from the tourist path, while halogen lights were located away from the path, next to geological formations and speleothems. The highest tourist flux is recorded in summer. Guided tours last more or less 60 minutes and each group is composed, on average, of 40 tourists, for a total of 700 tours per year. We therefore assumed that this cave is subjected, on average, to 700 illumination hours per year.

As the number of guided tours strictly relates to the number of lightning hours in the show cave, we considered the number of visitors as a proxy of lighting duration. Therefore, hereafter we refer to

Toirano (higher number of visitors) as “high pressure cave”, and to Valdemino (lower number of visitors) as “low pressure cave”. As the two caves are similar in terms of their geological substrate and microclimate, variations in the lampenflora proliferation among the two caves can be ascribed to the different lighting regime. It should be noted that the use of the number of visitors as proxy of lightning duration in the cave do not cover possible local small variations during guided tours of maintenance work. However, given our general aims and the different order of magnitude in the number of visitors between the two caves, it seems likely that such small differences are not causing any bias to the interpretation of our results. In both cases, the estimated yearly number of illumination hours is higher than the threshold of 100 hours per year suggested by Planina (1974) as the minimum number of illumination hours that is required during a year to allow the growth of the lampenflora.

2.2. Sampling design and data collection

We collected the data in June 2020. Samplings were performed on cave speleothems that have been never cleaned since the opening of the caves to tourists. We could thus assume that biofilms have reached their growth plateau. Indeed, we aimed at working on mature biofilms to avoid confounding effects caused by different colonization phases (Mulec et al., 2008). It should be noted that samplings were performed one month after the end of the national lockdown instituted by the Italian government to contain the Covid-19 pandemic. In that period, both show caves had been closed to the public for 90 days. However, as both caves have been closed and opened on the same date (March 9, 2020 and June 6, 2020), we assumed an equal effect (if any) in both cases.

To evaluate the presence and concentration of the lampenflora, we selected one illuminated speleothem (hereafter ‘plot’) on average every 50 m from the cave entrance along the tourist path of both caves for a total of 22 plots in Toirano cave (high light pressure cave) and 14 plots in Valdemino cave (low light pressure cave). We selected only speleothems illuminated by halogen lamps in order to guarantee comparable data from the two caves. As the presence of photosynthetic biofilms in show caves often depends on the bioreceptivity of the substrate, i.e. its potential to be colonized by different microorganisms (Gillieson, 1996; Miller, Dionísio, Laiz, Macedo & Saiz-Jiménez, 2009; Miller et al., 2012; Warscheid & Braams, 2000) we selected only bare rocks without sediments on their surface.

Each sampling point consisted in a circle of 20 cm diameter, placed on the illuminated area of the speleothems, where we measured three replicates of the density of the three main photosynthetic groups constituting the autotrophic component of biofilms in caves, namely cyanobacteria, green algae and diatoms. These measurements were performed with the BenthoTorch®, developed by BBE Moldaenke GmbH (Schwentinental, Germany). BenthoTorch® is a Pulse Amplitude Modulated (PAM) fluorimeter emitting light pulses at three different wavelengths (470, 525 and 610 nm), and recording the response of cyanobacteria, diatoms and green algae at 690 nm wavelength. Thanks to an inbuilt algorithm, the instrument calculates an instantaneous and in situ measure of the density of the three benthic photosynthetic organisms, which is expressed as chlorophyll-a (chl-a) concentration (µg chl-a/cm^2). The instrument discriminates the three groups on the base of the different pigments distinctive of the target groups (phycocyanin for cyanobacteria, chlorophyll c and xanthophylls for diatoms and chlorophyll b for green algae). A 700 nm LED is used to compensate the reflectance of the background (Carpentier et al., 2013). It must be emphasized that measurements obtained with the BenthoTorch® should not be intended as an indirect measure of algal biomass and biovolume, but only as a proxy of biofilm primary production (Kahlert & McKie, 2014). In addition, measurements obtained on thick mats or at chlorophyll-a values > 5 µg/cm^2 may be biased compared to standardized laboratory methods (Echenique-Subiabre et al., 2016; Kaylor, Argerich, White, VerWey, & Arismendi, 2018; Rosero-López, Walter, Flecker, Ontaneda, & Dangles, 2021) and chlorophyll-a measurements with the BenthoTorch® should be performed not in full sun but after a period of 20-30 minutes of low-light adaptation of biofilms (Kaylor et al., 2018). However, these problems do not occur in caves because: i) biofilms are extremely thin (< 1 mm); ii) chlorophyll-a values are generally low (< 1 µg/cm^2); and biofilms are constantly exposed to low light. Therefore, we assumed that measures obtained with the BenthoTorch® are representative of the real density values of the three examined groups.

To test for the possible effect of other environmental variables, in each plot we also measured light intensity with a DELTA OHM S.r.l. LP 471 PHOT probe and the distance from the lamp with a laser distance meter Disto Leica D110 BT (precision ± 1.5 mm; reach 0.2-60 m). We then provided a proxy of the water content by visually characterizing wetness at each plot. We assigned 0 to dry plots (no visible or perceivable wetness), 1 to plots with humid surface and 2 to plots with seeping water. The presence of seeping water was also interpreted as an indicator of nutrient availability and aquatic dissemination of microorganisms. The distance from the cave entrance was estimated by progressively measuring the distance between each sampling plot with the laser distance meter. This parameter was considered as a proxy of the microbial aerial diffusion, as speleothems located next to cave entrance are likely more easily colonized by microorganisms than those located deeper in the cave.

2.3. Data analysis

We retained the median value of the three chl-a replicates collected in each plot to be included in the subsequent statistical analyses. We preferred the median over the mean because it does not assume the distribution of values to be unskewed (Legendre & Legendre, 2012). Given that the distribution of the chl-a measures obtained for the three groups was extremely skewed towards low values (Fig. S1), we used the median values as dependent variable in the subsequent analyses in order to obtain robust and unbiased results. To obtain a measure of the total chl-a for each plot, we summed the chl-a medians obtained for cyanobacteria, diatoms and green algae. We then converted the cave identity into a measure of tourist use by assigning 1 to the low light pressure cave and 2 to the high light pressure cave and we considered this parameter as a proxy of the number of illumination hours. All statistical analyses were performed in the R environment (R Core Team, 2020).

First, we checked whether the overall light intensity measured at the different plots was comparable between the two caves with a Mann-Whitney U-test for non-parametric data. This preliminary test was performed to verify the assumption that possible differences between the two caves could be ascribed only to the different number of illumination hours, without any influence of the overall light intensity. We subsequently performed data exploration (Supplementary Material 2) in accordance with Zuur, Ieno, Walker, Savaliev & Smith (2009) and Zuur, Ieno & Elphick (2010). We checked the distribution of both dependent (Fig. S1) and independent (Fig. S2) variables to identify outliers and we tested for collinearity among covariates (Fig. S2) with the Pearson's correlation test (only variables with $|r| < 0.5$ were retained). After this check, we excluded the distance from the lamp from the subsequent analyses as it was collinear with the light intensity ($r = -0.5$). A log-transformation was applied to illuminance to achieve homogenization of its distribution.

We then tested the predictor variables, namely light intensity (light_intensity), distance from the cave entrance (distance_entrance), water presence (water) and tourist use (tourist_use), against the dependent variables via generalized linear models (GLMs, in accordance with Zuur et al. 2009). We performed four different models considering as dependent variables: the total chl-a concentration (1), the chl-a concentrations of diatoms (2), of cyanobacteria (3) and of green algae (4).

Considering that our dependent variables could not assume negative values, we adopted a Gamma distribution in our models, which was preferred over the Gaussian distribution, which allows prediction of negative values. This approach could be straightforwardly adopted with the total chl-a concentration data, which were strictly positive. When considering the response of the three photosynthetic groups, we applied Zero-Inflated Gamma models (ZIG) as suggested by Mills (2013) when data are characterized by a high presence of zero values in the dependent variables. Following this approach, for each photosynthetic group, we fitted two separate models: a binomial-GLM to test the probability that an outcome is a non-zero value, whose output allowed us to explain which covariates affect the presence or the absence of the target groups (namely the conditions determining a higher probability of colonization); and a gamma-GLM to deal with strictly continuous positive values, whose output allowed us to explain the role of covariates in determining values of chl-a density of each group. We ran the binomial model using a complementary log-log link function (clog-log), as recommended in Zuur et al. (2009) for datasets with unbalanced set of zeros (absences) and ones (presences), while we used a log link function for the gamma models.

3. RESULTS

Light intensity ranged from 4.06 to 340 lux (mean = 53.5 ± 70.4 lux) in the high light pressure cave, and from 4.35 to 280 lux (mean = 68.6 ± 73.8 lux) in the low light pressure cave. The results of the Mann-Whitney U-test applied to the light intensity recorded in the two examined caves showed no significant differences (*W* = 181; *n* = 36; *P* = 0.395), underlying how differences between the two caves can be ascribed exclusively to the different number of illumination hours.

The overall chl-a concentration showed, on average, higher values in the high light pressure cave than in the low light pressure one (Table S2). The results of the GLM (Tab. 2a), confirmed that this difference among the two caves is significant, with higher values in the high than in the low light pressure cave (Fig. 2a). In addition, we recorded a positive effect of light intensity (Fig. 2b), suggesting how these two parameters represent the main drivers of the abundance of photosynthetic microorganisms in show caves.

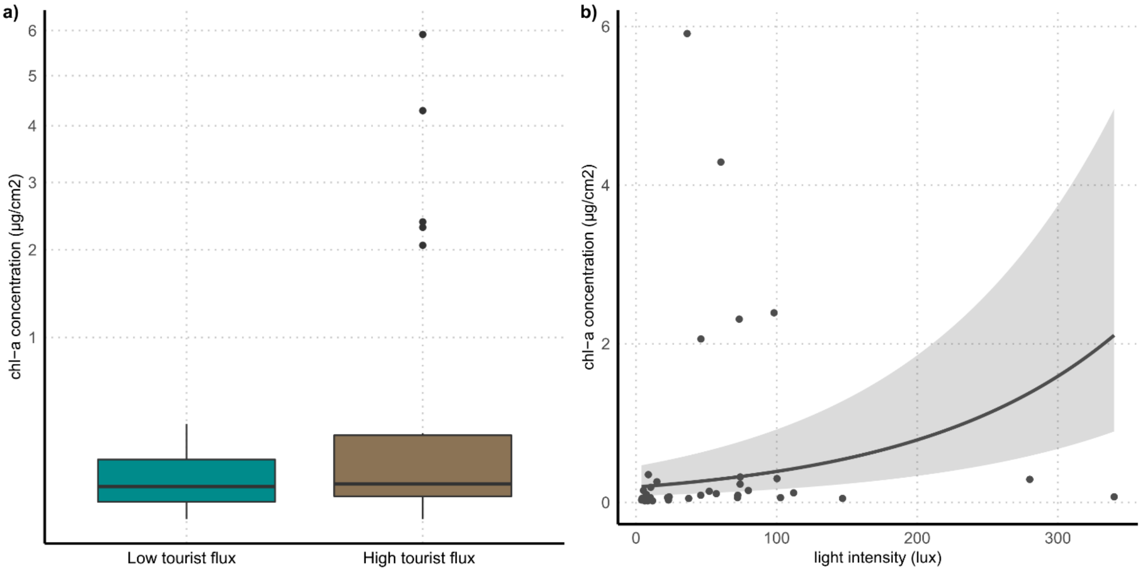

Figure 2. a) Boxplots representing total chl-a concentration in the low (light blue) and high (brown) pressure cave; b) predicted values and confidence intervals for the total chl-a concentration modelled against light intensity.

When analysing the patterns of the three photosynthetic groups, diatoms were dominant, being present in 33 out of 36 examined plots, followed by cyanobacteria that were present in 28 out of 36 plots. Green algae were the less represented group, being present only in 11 out of 36 plots, but they showed the highest concentration values (Tab. 1). The binomial models performed on the presence/absence values of the three photosynthetic groups (Table 2b) showed no significant effect of the environmental drivers here considered.

The chl-a concentrations of the three examined photosynthetic groups were in general extremely low, with diatoms being on average more abundant, followed by cyanobacteria and green algae (Tab. 1). The results of the GLMs performed on concentration values (Table 2c) showed a significant difference among the two examined show caves for all the three groups (Fig. 3a). In particular, we observed higher values in the high light pressure cave compared to the low light pressure cave, suggesting that longer periods of cave illumination increase the density of the three examined photosynthetic groups. In addition, results of the GLMs fitted with a gamma distribution also showed a positive effect of light intensity on cyanobacteria (Fig. 3c) and a nearly significant effect on diatoms (Fig. 3d), whose density is therefore enhanced on speleothems where the light intensity is higher. Finally, the presence of water significantly increased values of chl-a concentration of diatoms (Fig. 3b), suggesting a positive effect of seeping water on this group.

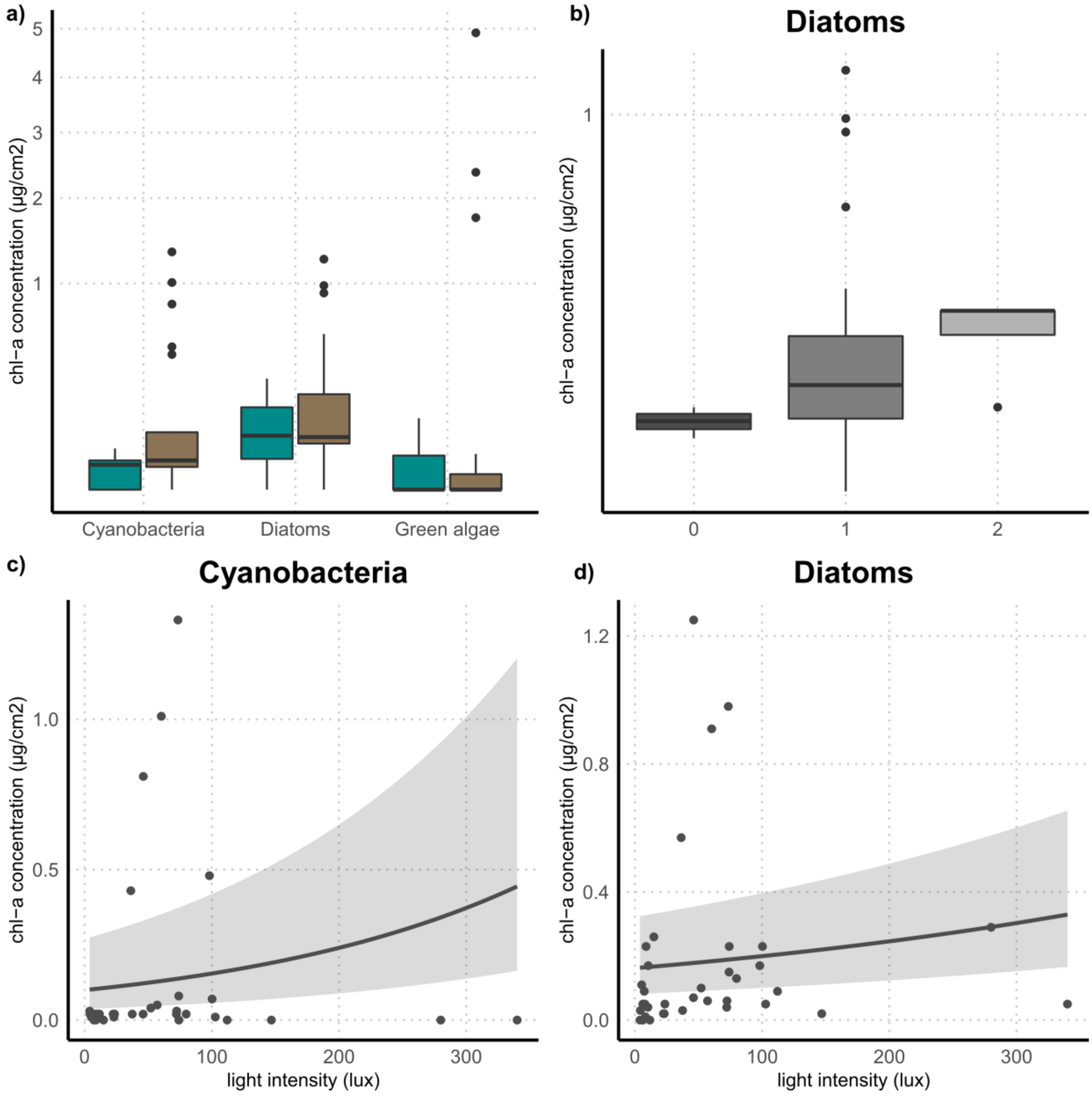

Figure 3. a) Boxplots representing chl-a concentrations of the three examined photosynthetic groups in the low (light blue) and high (brown) pressure cave; b) boxplots representing chl-a concentrations of diatoms in three different conditions of humidity (0 = dry rock ; 1 = moist rock; 2 = rock with seeping water) ; c) predicted values and confidence intervals for the chl-a concentration of cyanobacteria modelled against light intensity; d) predicted values and confidence intervals for the chl-a concentration of diatoms modelled against light intensity.

4. DISCUSSION

Lampenflora is considered a major threat for show caves, as it can heavily proliferate nearby artificial lights causing aesthetic damage and chemical alteration of cave speleothems (Albertano, 2003; Aley, 2004). Identifying the main environmental factors determining its presence and abundance is therefore a crucial step to identify the most vulnerable areas within show caves. Accordingly, appropriate management practices should be implemented to limit its growth and increase sustainability of tourism in caves. Light intensity has been recognized to be among the main drivers of the lampenflora growth, by positively affecting the proliferation of photosynthetic biofilms in show caves (Piano et al., 2015). Although the role of illumination duration on lampenflora growth has been widely recognized (Cigna, 2011; Planina, 1974), effective attempts to demonstrate statistically this positive effect are currently lacking in literature (but see Borderie et al., 2014).

By comparing two show caves sharing similar environmental conditions and management but different tourist use, we demonstrated that a higher tourist flux significantly enhances the overall proliferation of lampenflora. As the light intensity measured within the two show caves was fully comparable, we were able to ascribe this positive effect uniquely to the longer periods of illumination in Toirano cave, i.e. where the tourist flux was higher. The number of illumination hours was already suggested to be an important driver of the extensive growth of lampenflora by Planina (1974), and this hypothesis was confirmed by Borderie et al. (2014) who demonstrated that longer periods of illumination caused a higher lampenflora growth in Moidons cave (France). However, effective attempts to evaluate whether the length of the illumination period may differentially affect the different photosynthetic groups are currently lacking in literature. By separately analysing the response of cyanobacteria, diatoms and green algae, we could demonstrate that the effect of this parameter is consistent across the three groups, all showing significantly higher chl-a concentrations in the cave subjected to the highest tourist flux.

Besides the number of illumination hours, light intensity significantly increased the total chl-a concentration, confirming data obtained in literature (Mulec, 2014), but this effect was not consistent across the three groups, in contrast with Piano et al. (2015). In fact, the overall proliferation of the lampenflora was mainly driven by an increase in cyanobacteria and, to a lesser extent, in diatoms, but not of green algae. This discrepancy with literature data could depend on the examined range of light intensity, with values below 10 lux in 25% of sampling sites (9 out of 36 sampling plots) and below 40 lux in 50% of sampling sites (18 out of 36 plots). Johnson (1979) suggested that lampenflora algae require light intensities ranging from 10 to 50 lux to develop, while Cigna (2011) indicated that 40 lux represents the minimum value of light intensity that guarantees the development of the 85% of lampenflora organisms. We can thus assume that light intensity was below the minimum threshold to show a clear positive effect on the growth of eukaryotic algae, while the proliferation of cyanobacteria is favoured even by light intensities below 10 lux. This is in accordance with literature, as different authors showed that cyanobacteria can successfully grow even at light intensities considerably below the photosynthetic compensation point (Albertano, 2012; Bruno & Valle, 2017).

Besides the lighting regime, our results showed that also the presence of water significantly influences the lampenflora proliferation by favouring the growth of diatoms. This outcome is consistent with literature (Falasco et al., 2014; Nikolić et al., 2020; Padisàk, Rajczy, Paricsy-Komaromy & Hazslinszky, 1984; Roldán & Hernández-Mariné, 2009; St. Clair, Rushforth & Allen, 1981) and with previous data collected in the geographically close (< 50 km) Bossea show cave (Falasco et al., 2015), where light and the presence of water resulted important variables in determining diatom species assemblages. In particular, the presence of seeping water is expected to enhance the water reaction with limestone and the consequent release of Ca^{2+} ions, which are needed by diatoms to adhere to rock surface (Mulec, 2012). Our data also showed that, on average, diatoms dominated the examined biofilms in both caves, similarly to what was obtained in nearby (< 50 km) Alpine (Piano et al., 2019) and Mediterranean (Piano, Falasco & Bona, 2017) temperate streams. It is interesting to notice that the average values of the three photosynthetic groups we observed in subterranean mature biofilms are obtained in a tropical stream at the beginning of the biofilm formation, between 7 and 14 days of colonization (Rosero-López et al., 2021). We can thus hypothesize that the light- and nutrient-limited environmental conditions observed in caves naturally constrain the biofilm development compared to epigean aquatic ecosystems.

Regarding the effects of environmental variables on the presence of the three examined groups, we showed that their establishment was not significantly influenced by any of the factors included in the statistical models. The lampenflora establishment is mediated by the bioreceptivity of the substrate, i.e. its potential to be colonized by different microorganisms (Miller et al., 2009, 2012), that was not considered in our analysis. However, the two caves we examined are comparable in terms of geological substrate and we located our sampling plots only on bare rock to avoid confounding factors related to the nature of the substrate. It is therefore plausible that the occurrence pattern of the three examined photosynthetic microorganism groups is determined by a stochastic distribution as often occurs in microorganisms with passive dispersal (Mulec, 2019). After their installation, their growth is then strongly driven by the local environmental conditions, which may determine or not their extensive proliferation (Falasco et al., 2014; Martinez & Asencio, 2010). Although difficult to prove, exploitative competition is also expected to have a role, especially at the first colonization stages (Baquedano Estevez et al., 2019). For instance, fast-growing green algae, such as *Chlorella minutissima*, are usually the first colonizers in caves (Mulec et al., 2008; Nikolić et al., 2020) and they can survive even at extremely low values of light intensity (Czerwik-Marcinkowska, Wojciechowska & Massalski, 2015; Roldán et al., 2004). However, they could be rapidly outpaced by cyanobacteria and diatoms, which dominate the biofilms, where the environmental conditions become more favourable, e.g. higher light intensity and water availability (Roldán & Hernández-Mariné, 2009), and biofilms can reach their mature stage. However, other authors obtained opposite results, pointing out that cyanobacteria are pioneers, but they can be rapidly outcompeted by eucaryotic organisms in stable environment (Mulec et al., 2008). Specific studies dedicated to the evaluation of the colonization process in caves should be performed to shed light on this.

Overall, our work suggests that modulating the lighting regime can help in limiting the growth of photosynthetic biofilms in show caves. Although the effective removal of phototrophic biofilms should rely on the adoption of environmentally-friendly chemical methods (e.g. Meyer, Seale, Permar, & MacClary, 2017; Faimon, Štelcl, Kubešova, Zimák, 2003), the installation of low intensity lights and reducing the duration of guided tours can help in limiting the growth of lampenflora. Alternatively, visitors can be equipped with portable lights, thus avoiding the installation of artificial lights within the cave and consequently reducing the energy introduced in the subterranean ecosystem (Grobbelaar 2000; Mulec & Kosi 2009).

ACKNOWLEDGMENTS

We are grateful to Benedetta Baroni for her help during the field work. We also thank Marta Zunino and Cooperativa Arcadia for providing us the access to the caves.

FUNDING

This work was realized within the framework of the PRIN SHOWCAVE “A multidisciplinary research project to study, classify and mitigate the environmental impact in tourist caves” - code 2017HTXT2R, funded by the Italian Ministry of Education, University and Research.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHORS’ CONTRIBUTION

EP and MI set the lines of enquiry and designed the study. EP, GN and MI collected the data. EP and GN analyzed the data. EP led the writing of the paper. All authors reviewed the first draft of the paper and provided important improvements to the original text.

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SUPPORTING INFORMATION

Table S1. Average temperature (± standard deviation) for each sampling plot within each cave based on hourly collected data. Data were obtained with Thermochrone iButtons® left in the caves from June to November 2020.

Cave	Plot	Temperature
Toirano	01	17.397 ± 1.450
	02	14.537 ± 0.324
	03	14.659 ± 0.289
	04	14.460 ± 0.087
	05	14.797 ± 0.086
	06	14.696 ± 0.071
	07	14.918 ± 0.115
	08	15.054 ± 0.136
	09	15.246 ± 0.302
	10	15.246 ± 0.278
	11	14.435 ± 0.316
	12	15.400 ± 0.079
	13	15.570 ± 0.078
	14	15.460 ± 0.070
	15	15.840 ± 0.200
	16	15.663 ± 0.084
	17	15.689 ± 0.042
	18	15.767 ± 0.058
	19	15.879 ± 0.092
	20	15.870 ± 0.142
	21	15.731 ± 0.057
	22	15.869 ± 0.338
	Total	**15.372 ± 0.677**
Valdemino	01	15.502 ± 0.215
	02	15.398 ± 0.150
	03	15.358 ± 0.151
	04	15.150 ± 0.132
	05	15.558 ± 0.100
	06	15.402 ± 0.076
	07	15.179 ± 0.032
	08	15.300 ± 0.056
	09	15.284 ± 0.028
	10	15.501 ± 0.173
	11	15.593 ± 0.092
	12	15.249 ± 0.028
	13	15.127 ± 0.035
	14	15.309 ± 0.071
	Total	**15.351 ± 0.150**

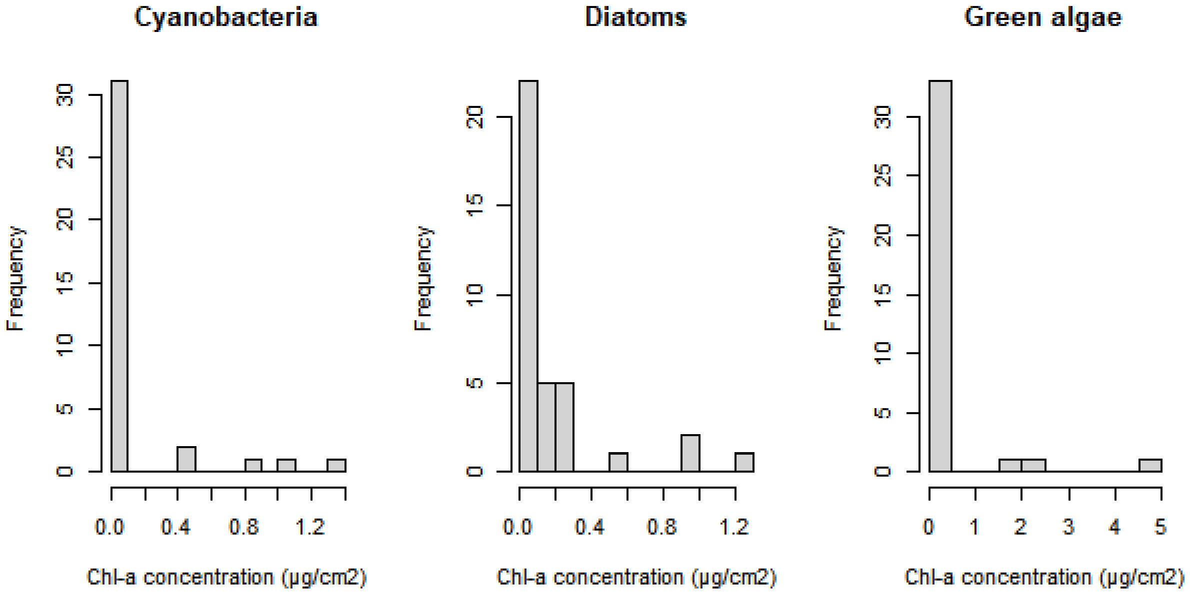

Figure S1. Histograms displaying the distribution of dependent variables (from left to right: chl-a concentration of cyanobacteria, diatoms and green algae).

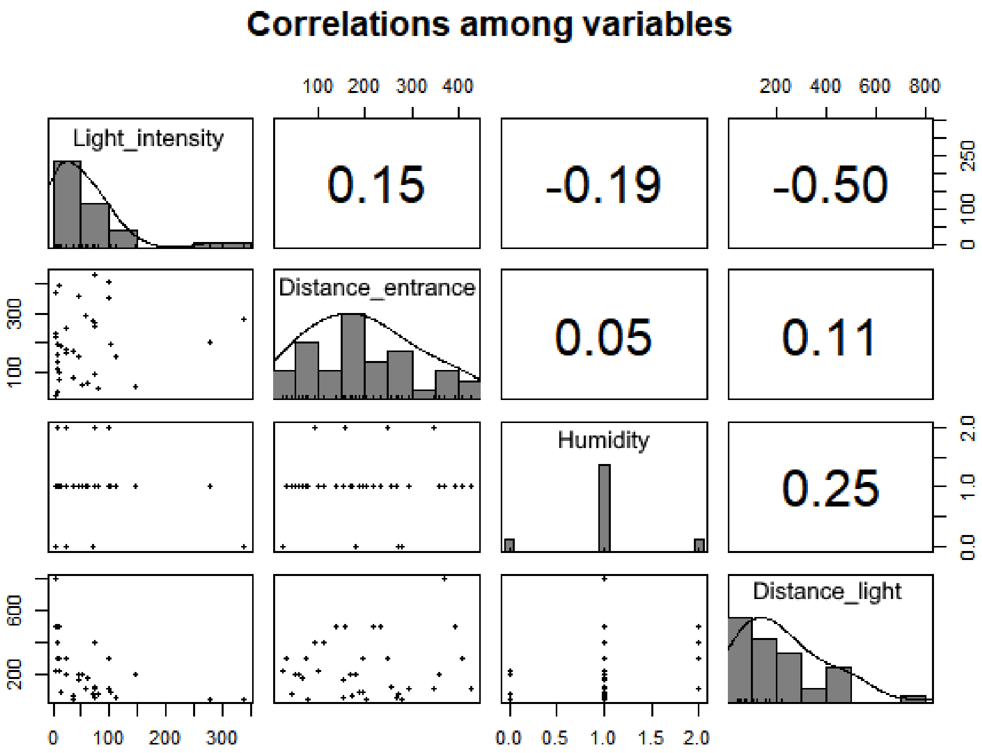

Figure S2. R Pearson correlation test applied to covariates in order to check for collinearity. We both checked the R correlation value (top right of the graph), any non-linear trend (bottom left of the graph) and variable distributions (diagonal). Light_intensity = intensity of light (lux); Distance_light = distance from the closest artificial light (cm); Distance_entrance = distance from the cave entrance (m); Humidity = moisture level of the plots.

Table S2. Summary of total chl-a concentration and chl-a concentration values for each photosynthetic group within the two show caves (High = high light pressure cave (Toirano show cave); Low = low light pressure cave (Valdemino cave). Data are expressed as mean and standard deviation (in brackets) of all samples and the ranges of the observed values are reported.

	Total chl-a		***Cyanobacteria***		***Diatoms***		***Green algae***	
	Low	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
Mean (±SD)	0.13 (±0.11)	0.85 (±1.59)	0.01 (±0.01)	0.20 (±0.38)	0.10 (±0.09)	0.24 (±0.36)	0.02 (±0.04)	0.41 (±1.2)
Range	0.02 – 0.35	0.02 – 5.91	0.00 – 0.04	0.00 – 1.33	0.00 – 0.29	0.00 – 1.25	0.00 – 0.12	0.00 – 4.91

Paper II

International Journal of Speleology | 50 (1) | 53-63 | Tampa, FL (USA) | January 2021

Available online at scholarcommons.usf.edu/ijs

International Journal of Speleology

Official Journal of Union Internationale de Spéléologie

Microhabitat selection of a Sicilian subterranean woodlouse and its implications for cave management

Giuseppe Nicolosi [1], Stefano Mammola [2,3], Salvatore Costanzo [4], Giorgio Sabella [5], Rosolino Cirrincione [5], Giovanni Signorello [6], and Marco Isaia [1*]

[1]Department of Life Sciences and Systems Biology, University of Turin, Via Verdi 8 - 10124, Torino, Italy
[2]Laboratory for Integrative Biodiversity Research (LIBRe), Finnish Museum of Natural History (Luomus), University of Helsinki, Pohjoinen Rautatiekatu 13, 00100 Helsinki, Finland
[3]Molecular Ecology Group (MEG), Water Research Institute, National Research Council of Italy (CNR-IRSA), Largo Tonolli 50, 28922 Verbania Pallanza, Italy
[4]Centro Universitario per la Tutela e la Gestione degli Ambienti Naturali e degli Agroecosistemi, University of Catania, Piazza Università, 2 - 95131 Catania, Italy
[5]Department of Biological, Geological and Environmental Sciences, University of Catania, Piazza Università, 2 - 95131 Catania, Italy
[6]Department of Agriculture, Food and Environment, University of Catania, Piazza Università, 2 - 95131 Catania, Italy

Abstract: Human activities in subterranean environments can affect different ecosystem components, including the resident fauna. Subterranean terrestrial invertebrates are particularly sensitive to environmental change, especially microclimatic variations. For instance, microclimate modifications caused by the visitors may directly affect local fauna in caves opened to the public. However, since numerous factors act synergistically in modulating the distribution and abundance of subterranean species, it remains challenging to differentiate the impact of human intervention from that of other factors. Therefore, evidence of the impact of tourism on cave invertebrate fauna remains scarce. Over a year and with approximately two visits a month, we investigated the effects of the presence of visitors on the subterranean endemic woodlouse *Armadillidium lagrecai* in the strict natural reserve of Monello Cave (Sicily, Italy). We found that natural microclimatic fluctuations, and not direct human disturbance, were the main factors driving the distribution of *A. lagrecai*. Specifically, *A. lagrecai* select for more climatically stable areas of the cave, where the temperature was constantly warm and the relative humidity close to saturation. We also observed a significant temporal effect, with a greater abundance of *A. lagrecai* in summer and a gradual decrease during the winter months. The number of visitors in the Monello cave had no effect on the abundance and distribution of *A. lagrecai*. However, considering the high sensitivity of the species to microclimatic variations, it seems likely that a significant increase in the number of visitors to the cave could indirectly affect this species by altering local microclimate. Constant monitoring of the environmental parameters within the cave is therefore recommended.

Keywords: Armadillidium, biodiversity conservation, cave arthropods, human impacts, subterranean fauna

Received 25 November 2020; Revised 3 March 2021; Accepted 3 March 2021

Citation: Nicolosi G., Mammola S., Costanzo S., Sabella G., Cirrincione R., Signorello G. and Isaia M., 2021. Microhabitat selection of a Sicilian subterranean woodlouse and its implications for cave management. International Journal of Speleology, 50 (1), 53-63. Tampa, FL (USA) ISSN 0392-6672 https://doi.org/10.5038/1827-806X.50.1.2370

INTRODUCTION

Subterranean ecosystems are highly stable relative to their surrounding surface environments (Poulson & White, 1969; Howarth, 1980; 1993). The permanent darkness, scarce energy inputs, constantly high relative humidity, and temperature stability exert a selective pressures driving the evolution of specific physiological, morphological, and behavioral traits on resident organisms (Culver & Pipan, 2010). Consequently, subterranean species often show pronounced sensitivity to even small changes in the environmental conditions (e.g., Barr & Kuehne, 1971; Howarth, 1980; Culver, 2005). Temperature is one of the major abiotic factors affecting subterranean species and their life history (Bernabò et al., 2011; Novak et al., 2014; Rizzo et al., 2015; Mammola et al., 2019b; Pallarés et al., 2019, 2020); most specialized subterranean species select microhabitats where the temperature is generally higher and stable, such as the innermost areas of caves. The presence of subterranean fauna is similarly correlated with stable and high relative humidity, as their thin cuticles of cave adapted organisms render them vulnerable

*marco.isaia@unito.it

to desiccation (Howarth, 1980) which they would encounter in the drier areas of the cave.

Owing to this sensitivity of the specialized subterranean fauna, there is nowadays a growing awareness of a need for protection of subterranean ecosystems (Mammola et al., 2019a).

As fragile and confined environments, cave ecosystems are susceptible to numerous impacts related to human activities (Culver & Pipan 2019; Romero, 2009). One of the most studied among these impacts is the effect of touristic activities in caves opened to visitors (Cigna, 2016). Human-induced impacts associated to tourist caves can affect different ecosystem components (Mammola, 2019), but our understanding of the nuances of these effects remains scarce (Mammola et al., 2020). A massive presence of tourist directly alters the underground microclimate resulting in seasonal variations in temperature and relative humidity (Cigna, 2004). At the same time, the joint effect of visitors and artificial lights affects the presence of microorganism and their distribution (Mulec, 2014). For example, organic materials passively transferred into the caves by visitors may enhance the growth of bacterial and fungal species alien to the cave community (Chelius et al., 2009; Mammola et al., 2017; Pfendler et al., 2018), whereas artificial lights lead to the development of photosynthetic biofilms (*lampenflora*) on rocky surfaces (Falasco et al., 2014; Piano et al., 2015; Baquedano Estévez et al., 2019). While an influence of tourism on the resident terrestrial fauna could also be expected, evidence related to the impact of direct anthropic disturbance on cave dwelling arthropods remains scarce (Isaia et al., 2011; Pellegrini & Ferreira, 2012; Pacheco et al., 2020).

Being specialized animals, subterranean invertebrates are theoretically vulnerable even to minor changes in their environment, including human-induced alterations. For instance, trampling of visitors and the subsequent modification to the substrate might cause a direct disturbance (Spate & Hamilton-Smith, 1991). The growth of *lampenflora* as well as the introduction of external opportunistic species of microbes favor the proliferation of more competitive species, which threaten the local cave-dwelling fauna (Mulec et al., 2008; Mulec & Kosi, 2009; Castello, 2014). Also, microclimate modifications associated with tourism could potentially cause the disappearance or migration of the local fauna (Slaney & Weinstein, 1997; Juberthie, 2000; Krajick, 2001; van Beynen & Townsend, 2005). A greater knowledge of the cave's main physical parameters inside the cave (in particular temperature, humidity, and CO_2) is essential for the conservation of the cave environment and its fauna. Moreover, is necessary to distinguish if environmental changes are due to natural causes or human activities in order to possibly define criteria for their conservation (Huppert et al., 1993; Fernandez-Cortes et al., 2006). However, due to the lack of long time series of data and several sampling impediments, it is generally difficult to tease apart the influence of these two factors in driving diversity patterns in caves (Eberhard, 2001).

The aim of our study was to explore the relative influence of microclimatic variations and the presence of visitors on the spatio-temporal dynamics of specialized subterranean fauna. We used the Monello Cave as a model system. It is located in a Strict Natural Reserve in eastern Sicily, and is renowned for its peculiar subterranean terrestrial fauna (Caruso, 1994). In particular, we focused on the specialized subterranean woodlouse *Armadillidium lagrecai* Vandel, 1969 (Malacostraca: Isopoda: Armadillidiidae) and its relation to both natural and anthropogenic factors related to cave tourism.

MATERIAL AND METHODS

Study area

The Monello Cave (cadastral number SiSr7007, Latitude 37°01'04.00" N; Longitude 15°09'57" E, altitude 10 m) is located in the municipality of Siracusa, in southeastern Sicily (Italy). The cave is included in the Strict Nature Reserve "Grotta Monello" (Fig. 1). The reserve was established in 1998 in order to protect the cave, its speleothems and its invertebrate fauna (see Caruso, 1994; Nicolosi et al., 2019; Sendra et al., 2019; Sabella et al., 2020). The cave is also part of the Special Area of Conservation (SAC) ITA090011 "Grotta Monello" and is formally recognized as a Geosite of regional interest. The site is managed by the Centro Universitario per la Tutela e la Gestione degli Ambienti Naturali e degli Agroecosistemi of the University of Catania (CUTGANA).

The cave is located in the eastern sector of the Hyblean Plateau, within the NW-SE trending Late Quaternary Floridia Basin (Ghisetti & Vezzani, 1980), a 12 km wide and 20 km long tectonic depression characterizing the northeastern sector of the Hyblean Plateau. It is located in the Early-Middle Miocene calcarenites and calcirudites with fragments of bryozoa, echinoderms, algae, and bivalves that constitute the upper member (or Siracusa Member) of the Climiti Mounts Formation (Pedley, 1981).

The cave has a total planimetric development of about 540 m. The first part consists of an artificially excavated horizontal gallery, 110 m in length, accessible through a small iron gate that prevents any unauthorized access to the cave. The gallery leads to the main room of the cave ("Sala Grande"). From there, several secondary passages originate, developing along different levels and showing a remarkable concentration of speleothems.

The cave was heavily modified to make it accessible for tourism. In the 80s, the Province of Siracusa excavated a pathway across the cave, which destroyed a huge number of speleothems (Fig. 2a).

Before the official opening, a study conducted by the University of Catania led to the discovery of an invertebrate assemblage of considerable interest (Caruso, 1994). This assemblage included specialized subterranean species such as the diplopod *Glomeris dionysii* (Strasser 1961) (Diplopoda: Glomerida: Glomeridae) and the isopod *Armadillidium lagrecai* (Fig. 2b), so far uniquely found in the Monello Cave. The peculiarity of the local fauna prompted the

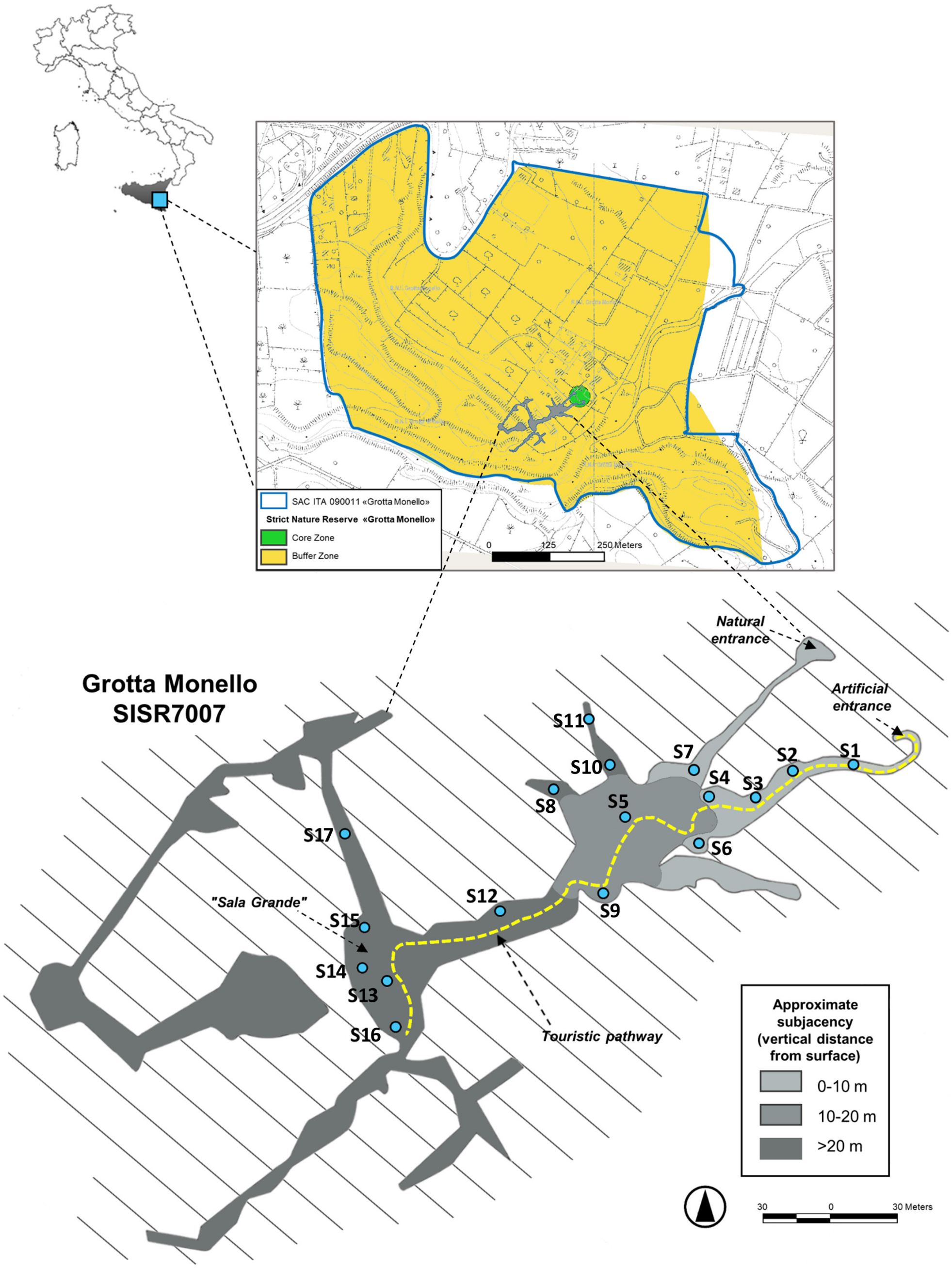

Fig. 1. Map of the study area. The sampling sites are represented by blue dots, located at 16, 24, 43, 47, 52, 62, 63, 73, 83, 84, 92, 96, 120, 123, 131, 136, and 143 m from the cave entrance. The different grey shades represent the subjacency (i.e., vertical distance from the surface).Topography of the Monello Cave is from the original drawings of Ruggieri & Amore (2000).

establishment of the Strict Nature Reserve, preventing uncontrolled touristic use.

Management of the Monello Cave

From its establishment in 1988 until 2018, access to the Monello Cave had been regulated by the managing body (CUTGANA) and its usage limited to approved scientific research.

Starting in 2018, the cave was opened to the public for a scientific investigation to evaluate the impact of visitors on the subterranean ecosystem. As of 2020, visitors were allowed in the cave under strict regulation: 3 visits were permitted per month and each visitor group was limited to twenty-four people, which in turn were divided in two subgroups of twelve, excluding one guide and one assistant. Visitors were not allowed to spend more than 40 minutes inside the cave, and each wore a helmet with a flashlight dimmer than 100 lumens. There was no built-in lighting system and visitors were required to stay on the visitor path.

Sampling design

We conducted the study from December 2018 to February 2020. We visited the cave 27 times over 14 months (approximately two visits/month).

Based on previous studies (Faille et al., 2014, Piano et al., 2015, Mammola et al., 2017), we considered the number of visitors and the distance from the tourist path as proxies for the human disturbance. We assumed these to be the only perturbations currently present in the Monello cave that are potentially capable of causing a measurable impact. In particular, we assigned to each sampling event the total number of visitors to the cave in the previous two weeks. In this regard, we selected nine sampling points along the visitor pathway (sampling points number 1, 2, 4,

Fig. 2. a) The corridor leading to the "Sala Grande" in the Monello Cave; b) The subterranean woodlouse *Armadillidium lagrecai* Vandel 1969; c) The cardboard used for sampling the fauna in the Monello Cave.

7, 9, 12, 13, 14, and 16), and eight further points away from the path and/or in the lateral branches not opened to public (sampling points number 3, 6, 5, 8, 10, 11, 15, and 17, see Fig. 1). We measured the distance from the cave entrance and from the pathway with a Leica DISTO (TM) laser distance meter.

In order to minimize impact on the fauna and avoid killing, we monitored the fauna using three sheets of corrugated cardboard, which were glued together using a starch-based glue to form single sheets of 15 cm height x 30 cm length. The presence of three sheets creates a thicker and longer lasting surface, otherwise, the high humidity of the cave would flake it quickly. The corrugated side was then placed on the ground (Fig. 2c). According to Caruso (1994), such a surface creates a microhabitat that attracts invertebrates, acting as a refuge, facilitating their count and eventual collection. We placed seventeen cardboard sheets within the cave.

The abundance of *Armadillidium lagrecai* under each cardboard was recorded at each sampling session. We also recorded the presence of *Glomeris dionysii*, *Chthonius (Chthonius) multidentatus* Beier 1963 (Arachnida: Pseudoscorpiones: Chthoniidae), *Roncus siculus* Beier 1963 (Arachnida: Pseudoscorpiones: Neobisiidae), *Tychobythinus inopinatus* Sabella, Costanzo and Nicolosi 2020 (Insecta: Coleoptera: Staphylinidae), and *Plusiocampa (Plusiocampa) tinoamorei* Sendra & Nicolosi 2019 (Entognatha: Diplura: Campodeidae) (see Table S1 and Fig. S1).

Being the main climatic factors known to affect the distribution of subterranean species (Tobin et al., 2013; Mammola et al., 2015; Kozel et al., 2019), at each sampling session, we used an Indoor Air Quality Monitor Delta Ohm instrument to measure instant temperature and relative humidity at each cardboard site. The outside air temperature was provided by SIAS (Servizio Informativo Agrometeorologico Siciliano).

Statistical analyses

We performed all statistical analyses in R (R Core Team, 2018). We explored the factors driving the spatial and temporal abundance of *Armadillidium lagrecai* within the cave using regression-type analyses (Zuur & Ieno, 2016). We calculated the standard deviation of temperature values during the whole sampling period and derived the subjacency (vertical distance from the surface) of each sampling point. We used abundance of individuals of *A. lagrecai* at each site as dependent variables. Given their low abundances, we could not fit regression models for the other species recorded in this study (Fig. S1).

We carried out data exploration following the protocol by Zuur et al. (2010). We evaluated the presence of outliers in the independent variables using Cleveland dotplots. We explored collinearity among the covariates using pairwise Pearson's r correlations, setting the threshold for collinearity at |*r*| ≥ 0.7. We explored non-linearity in the variable distributions and used coplots to evaluate the existence of potential interactions among covariates.

As a result of data exploration, we log-transformed the distance from the visitor pathway and square-root-transformed the total number of visitors in the previous two weeks to homogenize their distribution and cope with a few outliers. Pairwise Pearson's correlations revealed that the temperature was collinear with relative humidity (*r* = 0.76), standard deviation of temperature (*r* = –0.73), distance from entrance (*r* = 0.67) and subjacency (*r* = 0.62). The subjacency was also collinear with standard deviation of temperature (*r* = –0.89), and distance from the entrance (*r* = 0.88). Therefore, we kept temperature as the only independent variable in the analysis.

During data exploration, we found a non-linear response of the abundance of *A. lagrecai* to the sampling week (cumulative counting), reflecting a seasonal variability in the abundance of the species. In light of this seasonal variability and the relative association of the sampling week with temperature, we decided to i) model the temporal distribution of *A. lagrecai* separately using a generalized additive model; and ii) to include the sampling week as a random term in the main regression model, in order to study the effect of microclimatic conditions and tourism on the abundance of *A. lagrecai* avoiding the confounding effect of seasonality.

With respect to i), we fitted the generalized additive model using the *gam* command in the R package *gam* (Hastie, 1991). The structure of the model (in R notation) was:

y ~ s(Sampling week)

where y = abundance of *A. lagrecai* and s = smooth term.

With respect to ii), we modelled the abundance of *A. lagrecai* with a Poisson generalized linear mixed model (Zuur et al., 2009). We fitted the model in R using the *glmer* command in the R package *lme4* (Bates et al., 2015). The structure of the initial model (in R notation) was:

y ~ Temperature + Number of visitors + Distance from the path + (1| Sampling week)

where y = abundance of *A. lagrecai* and (1| Sampling week) is a random term to deal with repeated observations and measurements at each sampling site.

Once we fitted the initial models, we performed model selection with the R package *MuMIn* (Bartón, 2016) to select a best performing model the minimized AIC and maximized Aikaike weight values (Zuur et al., 2009). We carried out model validation on the final model using the R package *Performance* (Lüdecke et al., 2020).

RESULTS

Presence of visitors

During the monitoring period, the cave was visited by 953 visitors with an average of 68 visitors/month. Visitors were more abundant in spring between April and June 2019, with a peak of 159 visitors in June. Visitors were also abundant in November 2019 and January 2020. In contrast, the number of visitors was low in August and December, with 29 and 32 visitors, respectively (Fig. 3).

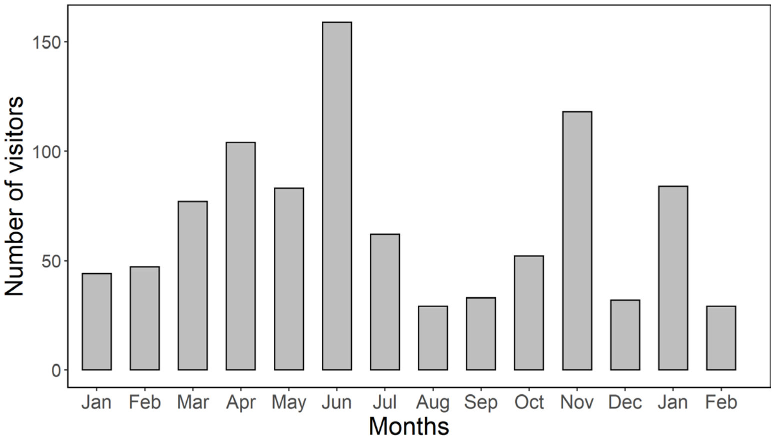

Fig. 3. Number of visitors/months during the monitoring activity.

Cave temperature and relative humidity

With regard to temperature, max–min ranges were attenuated with increasing distance from the cave entrance, and delayed compared to the values recorded outside the caves. The mean annual temperature values deep inside the cave (sites S13-S17) showed little variation over the year (mean ±SD = 18.4 ± 0.29). Conversely, temperature in the outermost sector (sites S1-S3) showed greater variability (mean ±SD = 14.8 ± 2.2). Here, temperature was relatively stable in summer, spring and autumn, while in winter temperature aligned with the outside temperature. The lowest temperatures were recorded in January (12.7°C). The annual trend of the average temperature for each site is shown in Fig. 4.

Relative humidity was constantly close to saturation in the deeper part of the cave, with values ranging from 98 to 100% during the whole sampling period. In the entrance zone, the relative humidity measured was around 80% and showed greater variability (annual range of 20%) (Fig. 5).

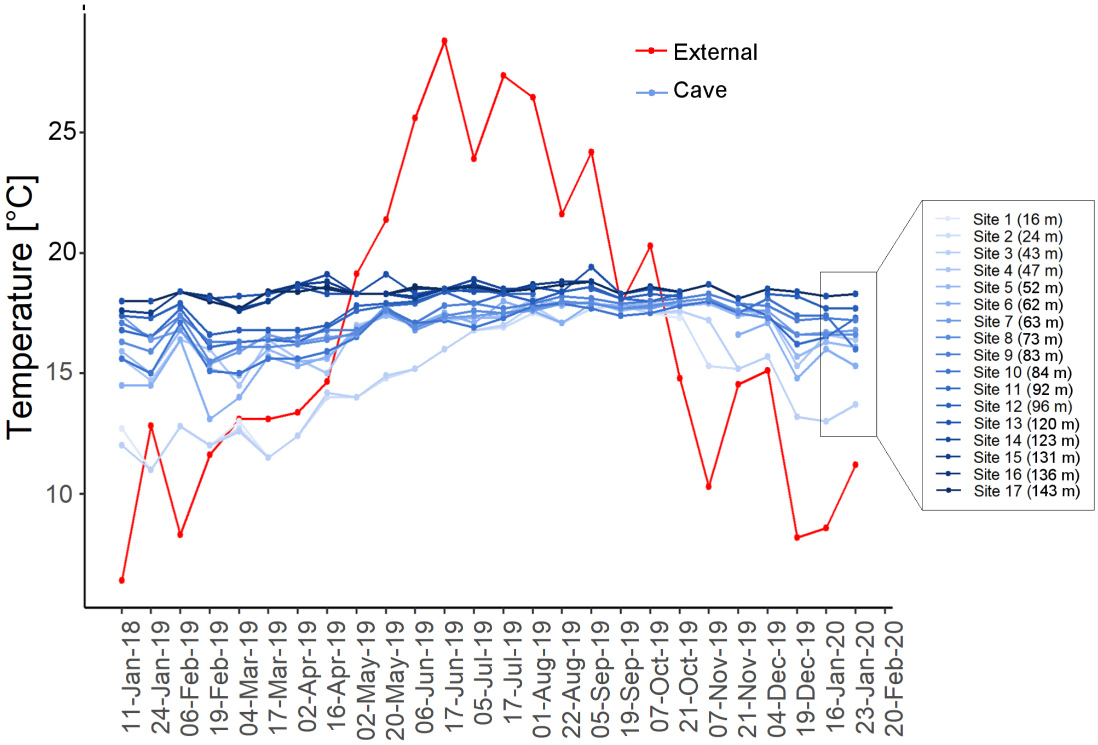

Fig. 4. Temperature of the Monello Cave. Annual trend of external temperature (red line) and temperatures inside the cave measured at each sampling site during the survey (blue lines). The shades of blues indicate the position of the sampling site relative to the cave entrance, from the outermost (lighter blues) to the innermost sites (darker blues).

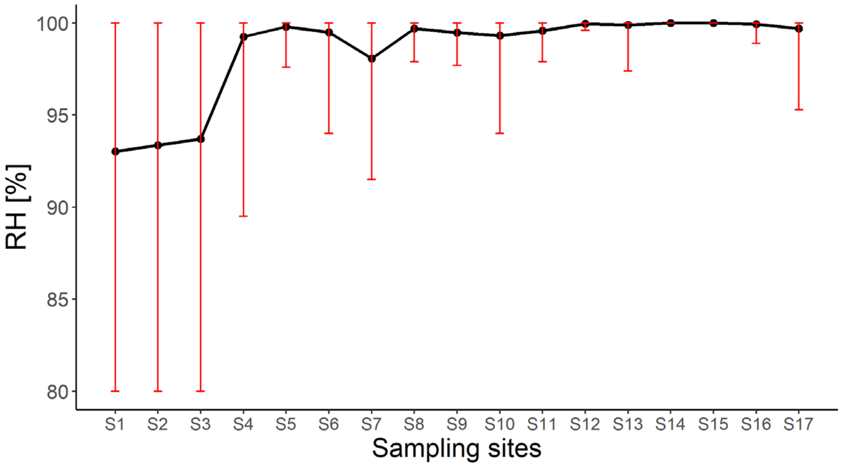

Fig. 5. Annual mean values of relative humidity with max-min ranges (red bars) for each sampling site.

Temporal distribution of *Armadillidium lagrecai*

The generalised additive model indicated that the abundance of *Armadillidium lagrecai* varied significantly throughout the sampling period (edf = 5.14; x^2 = 46.15, p <0.001), with a decrease from March to June and from November to January. In contrast, abundance increased in June and peaked in October (Fig. 6a).

Factors affecting the abundance of *Armadillidium lagrecai*

Results of model selection are shown in Table 1. The best performing model (Marginal R^2 = 0.77; Random effect variance ± s.d. = 0.11 ± 0.33) included the temperature and the distance from the visitor path as a fixed term. According to the model, species abundance significantly increased with increasing temperature, with the maximum abundance around 18°C (Fig. 6b). Given that temperature was positively correlated with relative humidity (r = 0.8), we recovered the same trend when we built the model by using the relative humidity instead of temperature (Fig. 6c), with *Armadillidium lagrecai* being more abundant in the sampling sites with relative humidity approaching or reaching saturation. None of the other factors had a significant influence on abundance, including the number of visitors and the distance from the tourist path. However, the latter was kept in the final model even though it was not significant based on the z-test (p = 0.06).

It is worth noting that the final model was slightly overdispersed (dispersion ratio = 2.1; Pearson's x^2 = 902.612; p <0.001). However, in light of the model validation (Fig. S2) and given that overdispersion ratio was rather low, we determined that the Poisson distribution was adequate and we did not switch to a quasi-Poisson or negative binomial distribution.

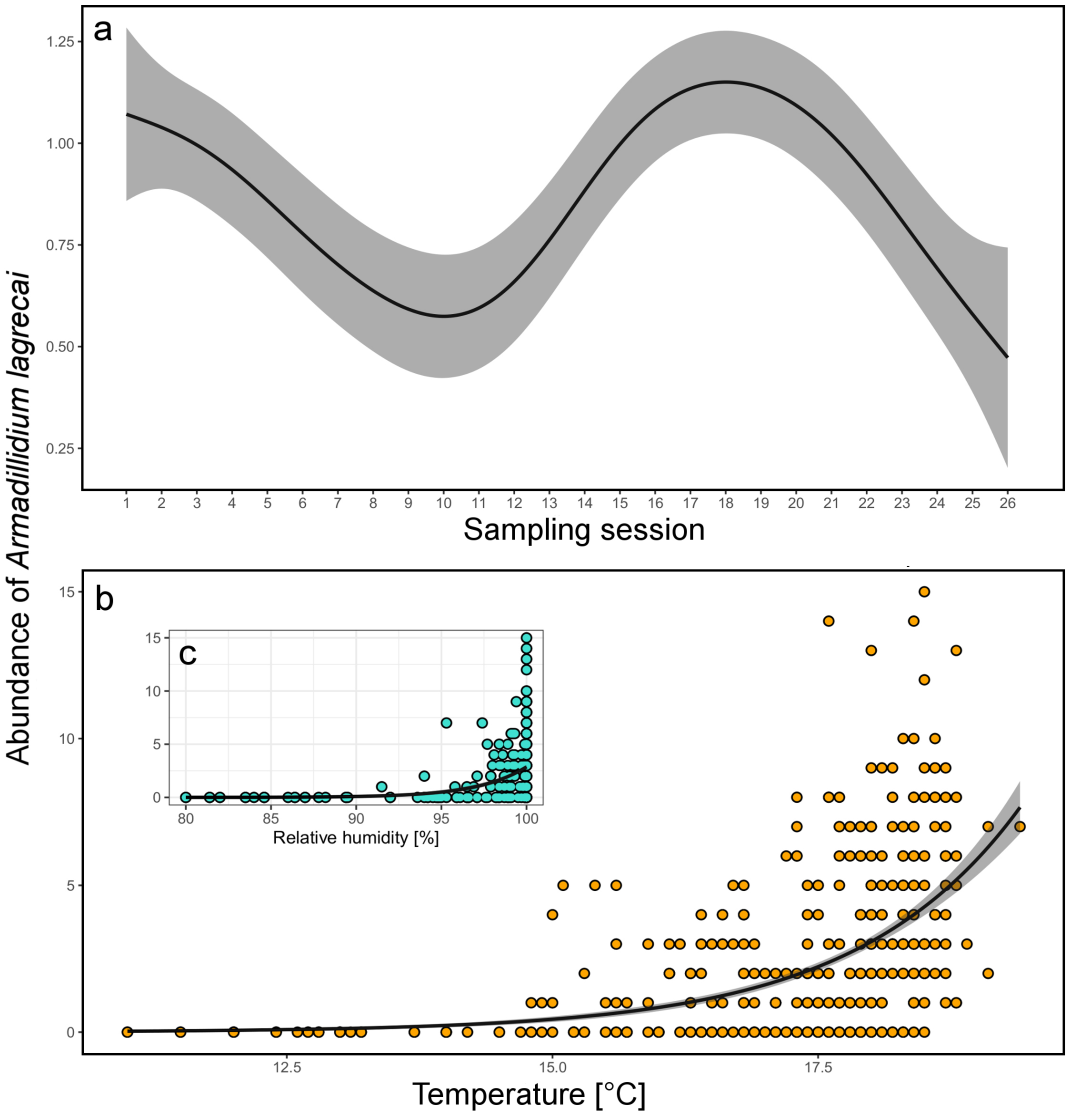

Fig. 6. a) Abundance trends of *Armadillidium lagrecai* in the Monello Cave. Generalized additive model analysis-predicted values (black line) and 95% confidence intervals (grey area) of abundance over time (smoother); b) Effect of temperature on the abundance according to a generalized linear mixed model. The inset graph (c) shows the trend obtained using relative humidity instead of temperature as explanatory variables in the model.

Table 1. Results of model selection and estimated regression parameters. Estimated regression parameters (Estimated β ± S.E.) for the fixed term are given only for the selected model. AIC = Aikaike Information Criterion; ΔAIC = (AIC of the model – AIC of the best model); df = Degrees of freedom; wi = Aikaike weights.

Model structure	**Variables**	**Estimated β ± S.E.**	**p-value**	**d.f.**	**AIC**	**ΔAIC**	**w_i**
y ~ Temperature + Distance from the path + (1| Sampling week)	Intercept	−12.32 ± 0.77	-	4	1776.56	0.00	0.67
	Temperature	0.74 ± 0.04	<0.001				
	Distance from the path (log-transformed)	0.05 ± 0.03	0.06				
y ~ Temperature + (1| Sampling week)		-	-	3	1777.95	1.39	0.33
y ~ Temperature + Number of visitors + Distance from the path + (1| Sampling week		-	-	6	2239.73	463.16	0.00

DISCUSSION

The opening of a cave to tourism activities impacts the cave ecosystem in several ways. The presence of visitors can lead the environmental alterations in terms of heat, airflow, humidity, and moisture (De Freitas, 2010), which potentially affects the resident fauna. Despite subterranean arthropod diversity in show caves could be high (see Culver & Sket, 2002), investigations aiming at unravelling the effect of tourism on cave arthropods remain scarce (e.g., Casale et al., 2008; Barciov et al., 2010; Fong, 2011; Isaia et al., 2011; Pellegrini & Ferreira, 2012; Faille et al., 2014; Pacheco et al., 2020).

By monitoring tourist access, it is possible to assess their effect on cave climatic stability, and eventually modify management strategies (Faille et al., 2014). For instance, monitoring activities should be carried out to evaluate the impact of human visitors on subterranean fauna and microclimate, especially if the cave falls within protected areas.

Although caves are stable environments, they exhibit seasonal changes in their microclimate which may modify the spatial and temporal distribution of subterranean species (Barr & Kuene, 1971; Peck, 1976; Howarth, 1980). These microclimate fluctuations are usually greater near the entrance, where the cave is most affected by external conditions, and drastically decrease in the deep areas where air temperatures remain stable, even when the external air temperature changes (Badino, 2010). Consequently, as in the Monello Cave, deep cave sectors emerge as optimal habitat for specialized subterranean fauna (Tobin et al., 2013; Mammola et al., 2015).

Based on the results of regression analysis, temperature and relative humidity seems to be more important than direct human impact in driving the distribution of *Armadillidium lagrecai* in the Monello Cave. Our data suggest that microclimate influences the spatial distribution of *A. lagrecai*, confirming its preference for the warmest and most stable cave sections, as well as an apparent seasonality effect on abundance variation which is possibly related to reproduction (Caruso, 1994).

The high correlation between temperature and relative humidity suggests the two factors play equally important roles in determining *A. lagrecai* abundance. The species showed a preference for areas of the cave where relative humidity approached or reached saturation (Fig. 6c). Relative humidity is considered to be one of the most important ecological limiting factors in the subterranean domain (Howarth, 1980; 1983), since even small variations can double the evaporation rate (Buecher, 1999) and have direct consequences on the resident fauna (Howarth, 1980). For example, subterranean woodlice possess cuticles with disproportionately thinner and more numerous endocuticular lamellae compared to their surface counterparts; the mechanisms preventing the evaporative loss of water are therefore reduced (Vittori et al., 2017).

Armadillium lagrecai abundance increased in summer (July–September) when temperature increased within the cave, and gradually decreased during the coldest months (October–January). This corroborates findings from Caruso (1994), who described a reproductive peak in May for the *A. lagrecai* when the warm season begins and cave temperatures gradually rise.

Armadillidium lagrecai demonstrated a clear preference for the innermost sampling sites of the cave (>96 m) where microclimate fluctuations were minimal. Moreover, considering the high correlation between the subjacency and both temperature and the distance from the cave entrance, we can infer that the abundance of *A. lagrecai* increased in the deeper zones with greater subjacency (>20 m). However, it was also in these innermost sections of the cave that we counted a lower number of specimens in the coldest months. This would suggest migrations toward the deeper voids and fissures of the cave, which according to Badino (2010) are characterized by even greater stability in terms of temperature fluctuation.

The distance from the visitor path was the only factor besides temperature that we kept in the analysis; however, its effect was not found to be statistically significant.

In accordance with previous studies (Faille et al., 2014; Pacheco et al., 2020), our analysis has not detected a strong direct effect of the presence of visitors on the abundance of our model species. In other words, with a moderate regime of 68 visitors per month, the current management of the Monello Cave does not seem to harm this subterranean species. The number of visitors present during the sampling activities, as well as the rigorous path delimitation, appears to minimize human disturbance. However, given the effect that a larger number of visitors may have on the cave microclimate, any departure from the current visitors regime we observed should be carefully assessed for the proper management of the cave.

CONCLUSIONS

The study investigated the factors affecting the microhabitat selection of *Armadillidium lagrecai.*

Under current management, it appears that the existing visitors regime in the Monello Cave has no effect on the abundance and distribution of *A. lagrecai*. Our data suggested that the species preferably select the areas of the cave where the microclimate factors were mostly constant during the sampling activities. The observed abundance fluctuations were determined by the microclimatic regime that occurs naturally inside the cave rather than by any disturbances caused by visitors.

However, any increase in visitor trampling could potentially cause the death of cave arthropods crossing the path or hiding under stones. Considering the moderate size of the cave, the number of visitors along the route should therefore continue to be scheduled, also limiting the permanence time in the “Sala Grande”, which correspond to the most microclimatically suitable area for the species.

Finally, it cannot be excluded that greater number of visitors could alter the cave microclimate in terms of temperature and humidity (Baker & Genty, 1998; Dominguez-Villar et al., 2010). Considering the high sensitivity of *A. lagrecai* to microclimate variations, and the fact that Monello Cave is one of the richest areas of troglobitic species in Sicily while being relatively small in size, the presence of visitors should continue to be accurately monitored with microclimatic surveys and visitor limitations maintained.

ACKNOWLEDGEMENTS

We are thankful to Jenny Liu for proofreading our English. This study was funded by Assessorato Regionale del Territorio e dell’Ambiente of the Regione Siciliana [Management Funds of Strict Nature Reserve “Grotta Monello” 2018, 2019]; SM is supported by the European Union’s Horizon 2020 research and innovation programme under grant agreement No. 882221. The research is included in the investigations conducted in the frame of "SHOWCAVE: a multidisciplinary research project to study, classify and mitigate the environmental impact in tourist caves", funded by Ministero Ministero dell'Istruzione and Ministero dell'Università e della Ricerca [PRIN: Progetti di ricerca di rilevante interesse nazionale 2017 - Prot. 2017HTXT2R].

Authorship statement: GN and MI set the lines of enquiry, designed the study and led the writing of the paper. GN wrote the first draft of the paper and prepared figures and tables. GN, SC, and GS set the sampling activities and collected the data. SM and GN analyzed the data. All authors reviewed the first draft of the paper and provided important improvements to the original text. This study is framed into a scientific investigation conceived by RC, SC, and GS aimed at evaluating the impact of visitors on the Monello Cave.

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Paper III

Microbial Ecology
https://doi.org/10.1007/s00248-023-02181-2

First Insights into the Bacterial Diversity of Mount Etna Volcanic Caves

Giuseppe Nicolosi[1,2] · José L. Gonzalez-Pimentel[3] · Elena Piano[1] · Marco Isaia[1] · Ana Z. Miller[3,4]

Received: 19 September 2022 / Accepted: 19 January 2023

Abstract

While microbial communities in limestone caves across the world are relatively understood, knowledge of the microbial composition in lava tubes is lagging behind. These caves are found in volcanic regions worldwide and are typically lined with multicolored microbial mats on their walls and ceilings. The Mount Etna (Sicily, S-Italy) represents one of the most active volcanos in the world. Due to its outstanding biodiversity and geological features, it was declared Natural Heritage of Humanity by the UNESCO in 2013. Despite the presence of more than 200 basaltic lava tubes, the microbial diversity of these hypogean systems has never been investigated so far. Here, we investigated bacterial communities in four lava tubes of Mount Etna volcano. Field emission scanning electron microscopy (FESEM) was carried out for the morphological characterization and detection of microbial features. We documented an abundant presence of microbial cells with different morphotypes including rod-shaped, filamentous, and coccoidal cells with surface appendages, resembling actinobacteria reported in other lava tubes across the world. Based on 16S rRNA gene analysis, the colored microbial mats collected were mostly composed of bacteria belonging to the phyla *Actinomycetota*, *Pseudomonadota*, *Acidobacteriota*, *Chloroflexota*, and *Cyanobacteria*. At the genus level, the analysis revealed a dominance of the genus *Crossiella*, which is actively involved in biomineralization processes, followed by *Pseudomonas*, *Bacillus*, *Chujaibacter*, and *Sphingomonas*. The presence of these taxa is associated with the carbon, nitrogen, and ammonia cycles, and some are possibly related to the anthropic disturbance of these caves. This study provides the first insight into the microbial diversity of the Etna volcano lava tubes, and expands on previous research on microbiology of volcanic caves across the world.

Keywords Bacteria · Microbial mats · *Actinomycetota* · Lava tubes · 16S rRNA gene analysis

Introduction

In the last 20 years, considerable effort has been made to shed light on the microbial communities of karst caves, particularly speleothems and walls hosting wall paintings [1, 2]. In contrast, the microbiology of lava tubes has received much less attention.

Microbial communities in lava tubes grow forming extensive colored biofilms on speleothems and walls [3], similarly to what happens in karst caves [4, 5]. Recent studies conducted in lava tubes revealed a highly diverse microbiome, dominated by new microbial life forms and interactions differing from those occurring on the surface [3, 6]. They can thrive in these harsh oligotrophic environments by interacting with minerals and inducing biomineralization processes [7].

When considering the composition of such colored microbial mats, evidence available in the literature showed that these colonies are mainly composed of metabolically active *Actinomycetota*, as revealed by cDNA analysis of yellow colonies from a lava tube in La Palma, Canary Island, Spain [8]. In general, *Actinomycetota* and *Proteobacteria* are the two most abundant groups of microorganisms in lava tubes (e.g., Northup et al. [9]; Hathaway et al. [10]). One of the most complete studies to date on *Actinomycetota* was carried out by Riquelme et al. [3], in volcanic caves from the USA, Canada, and Portugal, highlighting the importance of caves as a source of new species of *Actinomycetota* [3, 11].

✉ Ana Z. Miller
anamiller@irnas.csic.es

[1] Department of Life Sciences and Systems Biology, University of Turin, Turin, Italy

[2] Centro Speleologico Etneo, Catania, Italy

[3] HERCULES Laboratory, University of Évora, Évora, Portugal

[4] Instituto de Recursos Naturales Y Agrobiologia de Sevilla (IRNAS-CSIC), Seville, Spain

While we have some understanding on geomicrobiology of lava tubes from Hawaii, New Mexico and California, USA [6, 9, 10], Azores, Portugal [12], Easter Island, Chile [7, 13], British Columbia, Canada [3], Galapagos Islands, Ecuador [14–16], and La Palma Island, Spain [17, 18], no data are available on the microbial communities growing in lava tubes of Mount Etna (Sicily, S-Italy). Mount Etna represents one of the most active volcanos in the world [19], proclaimed a Natural Heritage Site by the UNESCO since 2013 due to its outstanding biodiversity and unique geological features. Its volcanic activity dates back more than 500,000 years ago. From about 57,000 years ago, the intense eruptive activity formed the 3600-m-high Ellittico stratovolcano, and from about 15,000 years ago, the Mongibello volcano, whose 357 lava flows cover 88% of the entire surface of Mount Etna [20].

Over 200 basaltic lava tubes are present around the Mount Etna volcano [21]. They generally form when the outer surface of lava channels cools more rapidly forming a hardened crust, while the inside continues to flow until it finishes draining and a tunnel is generated. The viscosity of the lava flow, which is related to the chemical composition of the molten rock, dissolved gases, temperature, and flow velocity, hence controls the formation of lava tubes [22]. The surface of “aa” lava flows is fragmental, spinose, and generally clinker, whereas “pahoehoe” lava flows are smooth. The formation of either is strongly influenced by the viscosity of the lava during eruptions [23]. Although it is believed that lava tubes only form on pahoehoe lavas, the formation of caves on Etna frequently occurs in “*aa*” lava, and some of the tubes that are considered to have formed on pahoehoe formed in large “*aa*” lava flows [24]. The composition of these caves corresponds mainly to basaltic lava, formed by silicates, such as clinopyroxene, plagioclase, and olivine, in addition to iron minerals, intercalated with carbonates and opal of biogenic origin [7, 13, 22].

Despite the great scientific interest determined by the frequent eruptive events occurring on the volcano, both on the summit and lateral flanks, and the peculiarity of its flora [25] and fauna (e.g., Caruso [26]; Magrini et al. [27]; Ebejer and Nicolosi [28]), research focusing on microorganisms is still lagging behind (but see Hopkins et al. [29]; Badalamenti et al. [30]), and study focused on microorganisms living on lava tubes has never been performed so far.

Here we aimed at providing the first microbiological assessment of biofilms coating the walls of four lava tubes located in Mount Etna Park (Sicily, Italy). The characterization of microbial communities from Etna lava tubes is fundamental to the understanding of this uncharted microbial diversity, and also contributes to the preservation of these unique geoheritage sites, recently receiving major interest from society both for their scientific and touristic value [31]. Therefore, reasonable protective and scientific measures should be applied to improve their value [32].

Materials and Methods

Studied Site and Sample Collection

The Etna volcano is located 20 km north of the city of Catania (15° 0′ E, 37° 43.8′ N), in Sicily, Italy (Fig. 1).

In June 2018, we performed a sampling campaign in four lava tubes of the Etna volcano, namely “Grotta del Santo,” “Grotta Catanese I,” “Grotta Lunga,” and “Grotta di Monte Corruccio.” The main features of each cave are reported in Table S1. Replicate samples of colored microbial mats were aseptically collected using sterile scalpels and stored in sterile 1.5-ml microtubes. Each microbial mat sample was collected from an area of approximately 20 cm^2. All samples were stored at 4 °C until transportation to the lab. Samples for DNA-based analysis were stored at − 80 °C until laboratory procedures were performed. Samples for microscopy observations were immediately processed upon arrival to the lab.

“Grotta del Santo” or “Grotta di San Nicola” (registered with reference SICT1032 at the “Catasto delle Grotte della Sicilia” of the “Federazione Speleologica Regionale Siciliana”) is a lava tube divided into several galleries reaching a total length of over 900 m. Lava flow here is attributed to a time interval of 15,000–3930 ± 60 years [20]. Results of bulk rock analyses for major (wt%) and trace (ppm) elements derived from Lanzafame and Ferlito [34] are presented in Table S1. The cave entrance has an altar erected in memory of Saint Nicola Politi, patron saint of Adrano (CT), who, according to tradition, lived in this place from 1134 to 1137. The cave is frequently visited, being a destination for religious pilgrimages (Fig. 1B). Although the presence of an iron gate, the cave is easily accessible. Within the lava cave galleries, four sampling sites comprising extensive yellow, white, and beige microbial mats coating the cave walls (designated GS_1A, GS_2A, GS_2B and GS_4; Table S2) were collected and analyzed.

“Grotta Catanese I” (SICT1037) is a lava cave that originated during the “Monte Arso” eruption, about 500 B.C. It is characterized by a large entrance hall, from which a narrow tunnel with about 70-m-long branches off laterally (Fig. 1C). The cave is freely accessible and occasionally frequented by local visitors. Grey and reddish colonies (designated GC1_1A and GC1_2; Table S2) were collected in the twilight zone.

“Grotta Lunga” or “Grotta di Monpeloso” (SICT1029) is a 55-m-long outflow tunnel that originated from the eruptive apparatus of “Monpeloso” formed in 300 ± 100 AD (Fig. 1D) [20]. Results of bulk rock analyses for major (wt%)

Fig. 1 Map of Mount Etna (Sicily, Italy) with the location of the sampled lava tubes (**A**). Source: Google Maps [33]. **B** Entrance of "Grotta del Santo." **C** Entrance of "Grotta Catanese I." **D** General view of "Grotta Lunga." **E** General view of "Grotta di Monte Corruccio"

and trace (ppm) elements derived from Matteoni [35] are presented in Table S1. Here we observed white and grey colonies (designated GL_1 and GL_3) along the main gallery (Table S2). The cave is freely accessible and frequently visited by tourists.

"Grotta di Monte Corruccio" (SICT1056) is an outflow tunnel partially contained in the effusive eruptive apparatus of the homonymous mount formed in a time interval of 15,000–3930 ± 60 years [20]. It has a total length of about 80 m (Fig. 1E). The cave is freely accessible and occasionally frequented by tourists. Here we observed yellow, white, and beige colonies (designated GMC_1, GMC_2, GMC_3, and GMC_4) along the cave galleries (Table S2).

Despite no information is available on the precise number of tourists entering each cave, based on an estimate of the tourist frequentation and the presence of waste due to visits, we can sort the four caves in two categories: those that experience low tourist use ("Grotta Catanese I" and "Grotta di Monte Corruccio") and the ones experiencing higher tourist use ("Grotta del Santo" and "Grotta Lunga").

Morphological Characterization by Microscopy Techniques

Small fragments of each sample collected in the four lava tubes were observed using a Zeiss Discovery V8 stereomicroscope (200 × maximum magnification) coupled to a MOTICAM 10.0 system to perform a detailed macroscopic examination of the sample surface.

Subsequently, samples were examined by field emission scanning electron microscopy (FESEM) using a high-resolution FEI Teneo SEM (FEI Company, Eindhoven, The Netherlands) equipped with an Oxford X-ray energy dispersive spectroscopy (EDS) detector for characterizing the microtopography of the samples and detect microbial cells.

Air-dried bulk samples with microbial mats were directly mounted on a sample stub and sputter coated with a thin platinum film, with an acceleration voltage of 5 kV, using the SE detector.

Statistical Analysis

The statistical analyses were performed in R [36]. The taxonomic richness measured at the order level for the four caves was tested by means of a generalized linear model (GLM) with a Poisson error distribution [37]. Differences among caves were then tested with the Tukey's post hoc test, with the function "glht" from the multcomp package [38].

Taxonomic Characterization of Microbial Communities

Molecular biology techniques based on 16S rRNA gene analysis were conducted for the identification of the bacterial communities present in the Etna lava tube samples.

Genomic DNA was extracted from 12 samples collected in the Etna lava tubes using the DNeasy PowerLyzer PowerSoil Kit according to the manufacturer's protocol (Qiagen), and quantified using a Qubit 4.0 fluorometer (Invitrogen). Sequencing libraries of the V3-V4 hypervariable region of the 16S rRNA gene were prepared according to the Illumina 16S Metagenomic Sequencing Library protocols. The gDNA input (2 ng) was amplified by PCR using the universal primer pair 314F (5′- Illumina overhang- CCT ACGGGNGGCWGCAG -3′) and 805R (5′- Illumina overhang- GACTACHVGGGTATCTAATCC -3′) with Illumina adapter overhang sequences. The thermocycling conditions were as follows: 3 min at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by a 5-min final extension at 72 °C. The PCR products were then purified with AMPure beads (Agencourt Bioscience, Beverly, MA). Two microliters of each purified product was PCR amplified for final library construction containing the index using NexteraXT Indexed Primer, under the same thermocycling conditions as mentioned before, except for 10 cycles. After purification with AMPure beads, the final purified products were quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantificatoin kits for Illumina Sequecing platforms) and qualified using the TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). The purified amplicons were then sequenced using the MiSeq™ platform (Illumina, San Diego, USA) by Macrogen Sequencing Services (Korea).

The raw data obtained from Illumina platform MiSeq, for producing 300 PE reads, was initially quality checked, trimmed, and clustered in ASVs using QIIME2 [39] with DADA2 [40]. ASV is a higher-resolution analogue of the traditional OTU table, which records the number of times each exact amplicon sequence variant was observed in each sample. Taxonomic identification was carried out using SILVA database v.132. Alpha diversity metrics (Shannon's index and Pielou's evenness) of bacterial communities were also calculated to investigate community heterogeneity within sample diversity. The raw reads wear deposited into the NCBI Sequence Read Archive (SRA) database under the project id PRJNA914266.

Results and Discussion

Microscopy Observations

Under the stereomicroscope, samples showed colored stains coating the rock substrate or associated with mineral grains (Table S2). There were notable differences in colony morphology, texture, and size.

Microbial cells and structures were imaged by high-resolution FESEM, as an effective and fast method for microbial life detection in complex samples. FESEM images showed abundant microbial cells with different morphotypes in all the samples (Figs. 2, 3, 4, and 5). The most common forms were rod-shaped and coccoidal cells with surface appendages, resembling the actinobacterial cells reported by [3] in lava tubes from USA, Canada, Portugal, and Spain. Samples from "Grotta del Santo," GS_1A (Fig. 2A, B, C), GS_2A

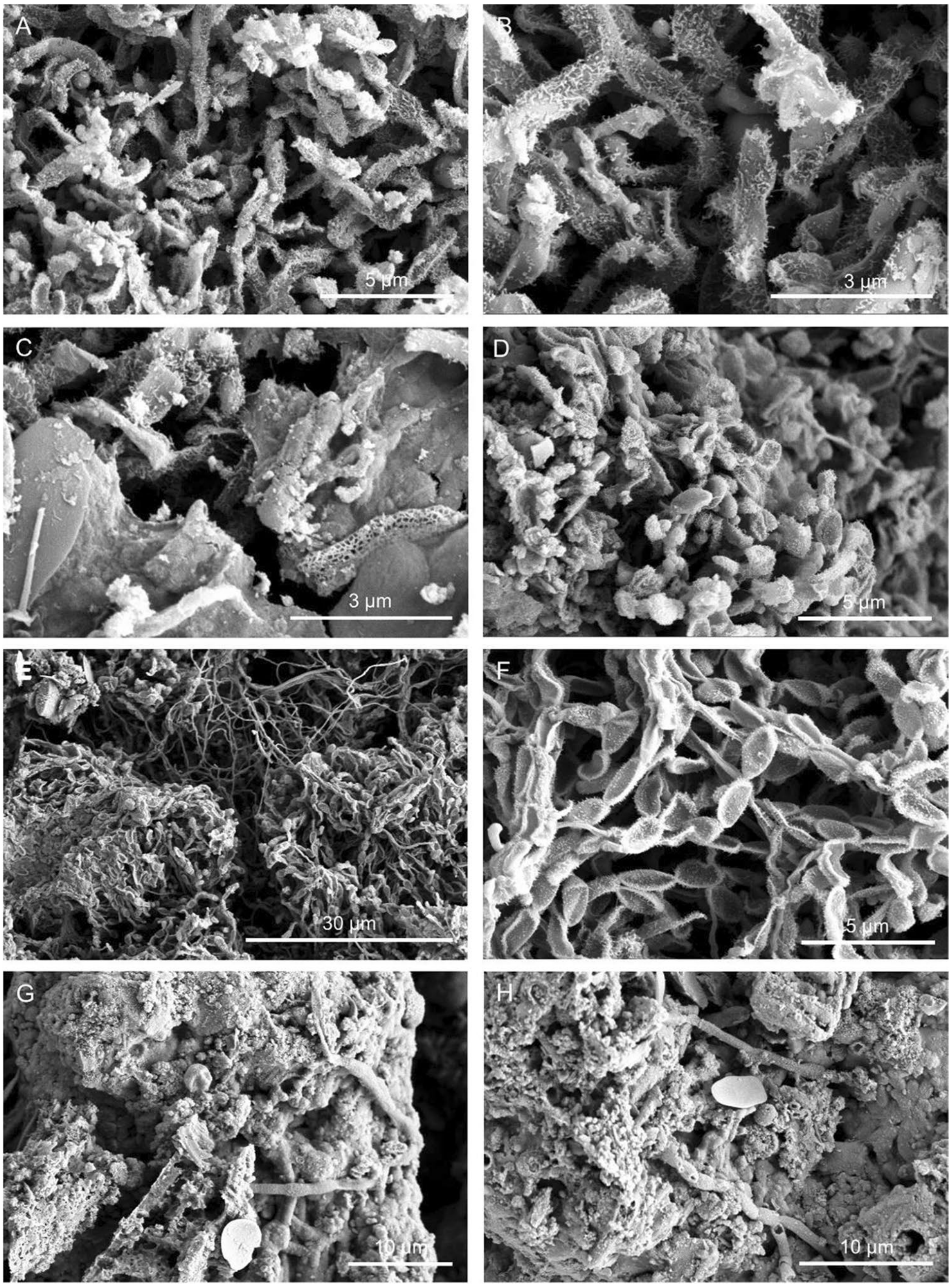


Fig. 2 Field emission scanning electron microscopy images of "Grotta del Santo" (GS) samples. Representative FESEM images of the studied samples, depicting **A** biofilm of filamentous actinobacteria-like cells (GS_1A); **B** close-up view of the spore chains with cell surface appendages (GS_1A); **C** fragments of reticulated filaments sparsely distributed within the sample (GS_1A); **D** biofilm of actinobacterial cells with spiny ornamentation (GS_2A); **E** dense network of actinobacteria cells with spiny ornamentations intermingled with Actinobacteria-like hyphae, and **F** spore chains with spiny protuberances on their surface (GS_2B); and **G**, **H** filamentous microbial structures with smooth surfaces in close association with the mineral substrate (GS_4)

(Fig. 2D), GS_2B (Fig. 2E, F), GC1_1A (Fig. 3A, B), and from "Grotta di Monte Corruccio," GMC_5 (Fig. 4G, H) showed to be the most abundant in actinobacteria-like cells, as revealed by FESEM.

FESEM observations showed an abundant presence of actinobacteria-like cells for the four samples collected in "Grotta del Santo," mainly comprising spores with a hairy surface (Fig. 2A–C) or chains of spores with spiny surfaces (Fig. 2D–F). Actinobacteria have been frequently reported in karstic and volcanic caves worldwide (e.g., Riquelme et al. [3]; Porca et al. [41]; Axenov-Gribanov et al. [42]). These bacteria grow from the hyphal, which is an important basis for classification and comparable to filamentous fungi [43]. Other filamentous bacterial structures with smooth surfaces were also found, particularly in sample GS_4, in close association with the mineral substrate (Fig. 2G, H).

Sample collected in "Grotta Catanese I" (GC1_1A) revealed the presence of several actinobacteria-like coccoid (1-µm diameter) and rod-shaped cells (Fig. 3A) with spiny ornamentation, as well as filaments with hairy ornamentation, intermingled with coccoid cells (Fig. 3B). In contrast, sample GC1_2 from the same cave showed a prevalence of diatoms, specifically *Orthoseira roeseana* (Fig. 3C) and *Humidophila* (Fig. 3D), which is related to its location near

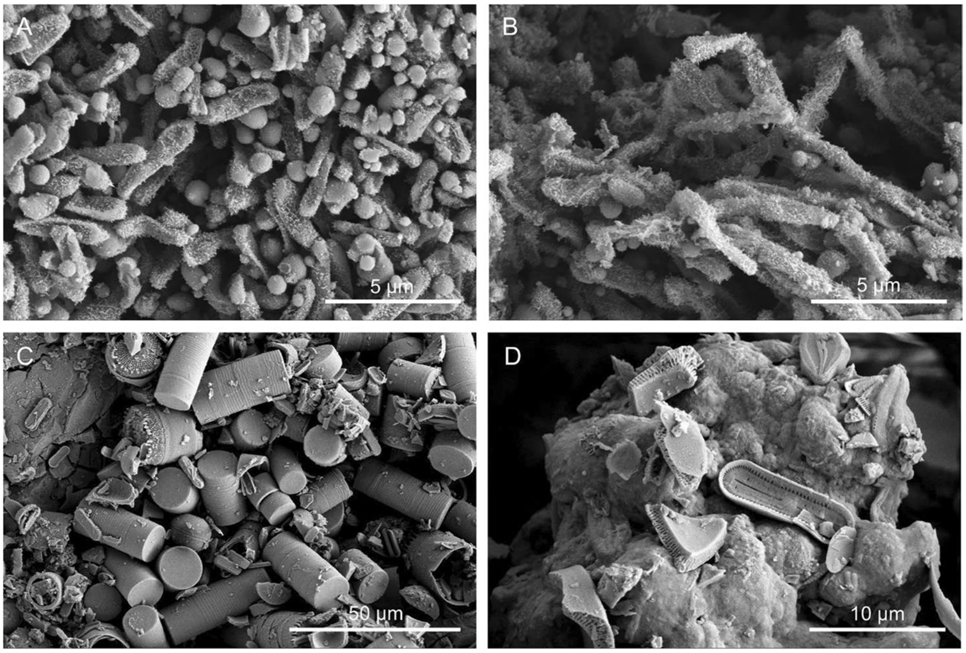


Fig. 3 Field emission scanning electron microscopy images of "Grotta Catanese I" (GC) samples. Representative FESEM images of the studied samples, depicting **A** Actinobacteria-like coccoid (with 1-μm diameter) and rod-shaped cells with spiny ornamentation (GC1_1A); **B** filaments with hairy ornamentation, intermingled with coccoid cells (GC1_1A); and **C**, **D** clusters of the diatom *Orthoseira roeseana* and *Humidophila* (GC1_2)

the cave entrance. It is well documented that at the entrance of caves and on artificially illuminated cave walls, phototrophic organisms mainly cyanobacteria, green algae, and diatoms develop with increasing moisture [44]. In this study, samples of whitish, beige, grey, or yellow bacterial mats that often form on the middle or dark zones of caves were selected and collected, tentatively avoiding phototrophic-based biofilms located at the cave entrances.

Samples collected in "Grotta di Monte Corruccio" showed greater variety in microbial structures and cell morphologies (Fig. 4). In the yellow mat sample (GMC_1), a biofilm of rod-shaped cells, embedded in a matrix of extracellular polymeric substances (EPS) was clearly observed by FESEM (Fig. 4A). Biofilms are multicellular microbial populations that typically adhered to solid surfaces due to the release of EPS, which self-encapsulate the cells and provide structure to biofilms [45]. They comprise a survival strategy of microbial cells to thrive in these hostile environments. Actinobacteria-like spores with hairy ornamentation and spiny surfaces were also observed in all samples collected in this cave (Fig. 4B–H). Sample GMC_4 revealed the presence of microbial imprints, suggesting that the cell-like shapes occur within internal laminae (Fig. 4E), as well as the presence of some clusters of Actinobacteria-like cells and rod-shaped bacteria impregnated in EPS (Fig. 4F). A tangled mass of actinobacteria-like hyphae or archaeal-like cells with hami that protrude from their cell surfaces was observed by Perras et al. [46].

Observations conducted on sample GMC_5 revealed the presence of Ca-rich spheroids closely associated with filamentous cells (Fig. 4G, H), resembling the $CaCO_3$ microspheres found in Kipuka Kanohina lava cave in Hawaii, USA [3]. Studies on cave microbial communities revealed that actinobacteria may induce biomineralization processes, promoting nucleation sites or changes in pH [3, 47]. Precipitation of carbonates can be induced by bacteria via urea hydrolysis [48] or through the uptake of carbon dioxide promoting changes in pH and followed by mineral precipitation and $CaCO_3$ formation [47]. Numerous biogenic minerals have been reported also in lava caves. Calcite and Mg–silicate minerals were found associated with actinobacterial morphologies on coralloid type speleothems from Chile [7]. Calcium carbonate spheres closely related to dense networks of hairy filaments were observed within the colored microbial mats from Azorean, Canarian, and Hawaiian [3]. Silica microspheres embedded in EPS matrix were observed in Galapagos Islands [16].

Interestingly, sample from "Grotta Lunga" (GL_1) showed the presence of reticulated filaments embedded in a matrix of EPS (Fig. 5A, B). The presence of reticulated filaments has been frequently reported in caves worldwide, in both lava and limestone caves [49–52] but their nature still results enigmatic for microbiologists [54]. FESEM observations of the sample GL_3 revealed a variety of actinobacteria-like filamentous forms spread all over the sample (Fig. 5C, D).

Richness and Diversity of Cave Microbial Communities

We identified a total of 6310 unique ASVs for the 12 samples, with the samples collected in "Grotta Lunga" being richer than the others (GL_3 = 1190 ASVs; GL_1 = 1057 ASVs). These samples also showed the highest values in

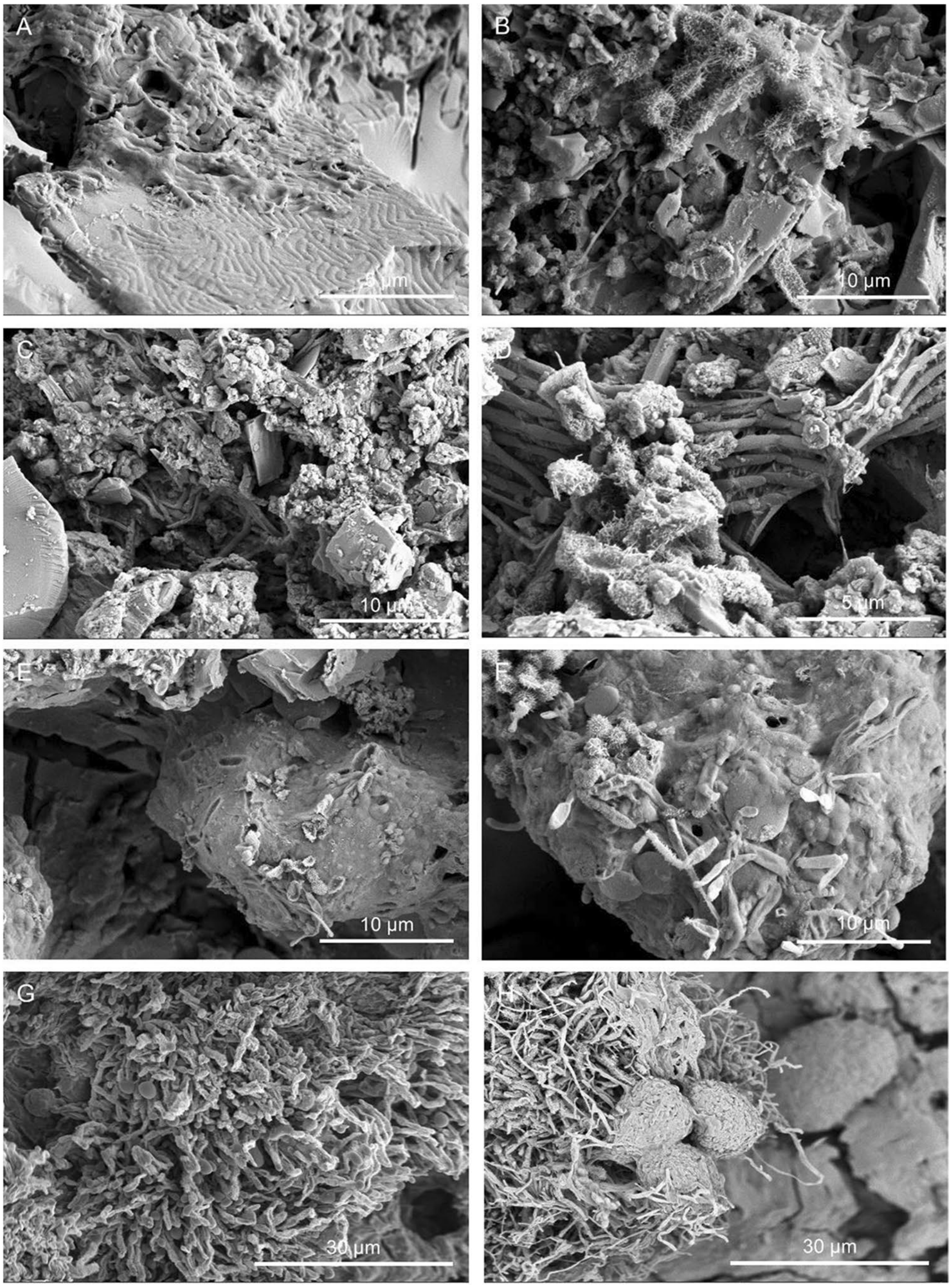


Fig. 4 Field emission scanning electron microscopy images of "Grotta di Monte Corruccio" (GMC) samples. Representative FESEM images of the studied samples, depicting **A** rod-shaped cells embedded in the EPS layer (GMC_1); **B** actinobacteria-like spores with hairy filamentous forms (GMC_1); **C** filamentous cells associated with mineral grains (GMC_2); **D** Actinobacteria-like cells with appendages (GMC_2); **E** microbial imprints showing cell-like structures (GMC_4); **F** clusters of Actinobacteria-like cells with spiny ornamentation (left) and rod-shaped bacteria with spiny ornamentation impregnated in EPS (right) (GMC_4); **G** dense masses of bacterial cells, mainly rod-shaped mats showing filaments covered with pili/fimbrae with spheroid shapes emerging from the ends (GMC_5); and **H** Ca-rich spheroids closely associated with filamentous cells (GMC_5)

terms of alpha diversity indices, i.e., Shannon index [54] and Pielou's evenness [55] (Table 1), calculated on the ASVs (Fig. 6).

When considering the taxonomic richness measured at the order level, the "Grotta Lunga" resulted the richest cave with 174 orders, followed by "Grotta Catanese I" with 150 orders, and then by "Grotta del Santo" and "Grotta di Monte Corruccio" caves with 148 and 147 orders, respectively (Fig. 7). The result of the GLM showed a significant effect of the cave identity on the taxonomic richness measured at the order level (Chi = 46.4; $P < 0.001$). By comparing the differences among caves with the Tukey's post hoc test, we could highlight that most of this effect is due to the significant difference between the "Grotta Lunga" and the others, with the former being significantly richer than the others (Lunga – Catanese: $z = 2.72$, $P = 0.034$; Lunga – Santo: $z = 6.41$, $P < 0.001$; Lunga – Corruccio: $z = 5.59$, $P < 0.001$). Regarding the other pairwise comparisons, we could record a significant difference between "Grotta Catanese I" and "Grotta del Santo" ($z = 3.15$, $P = 0.009$), while no other significant differences were identified ($|z| < 2.34$; $P > 0.05$).

Due to the heterogeneity of the examined caves, the small number of samples and considering the pioneer nature of this study, specific patterns are hard to discern, as well as

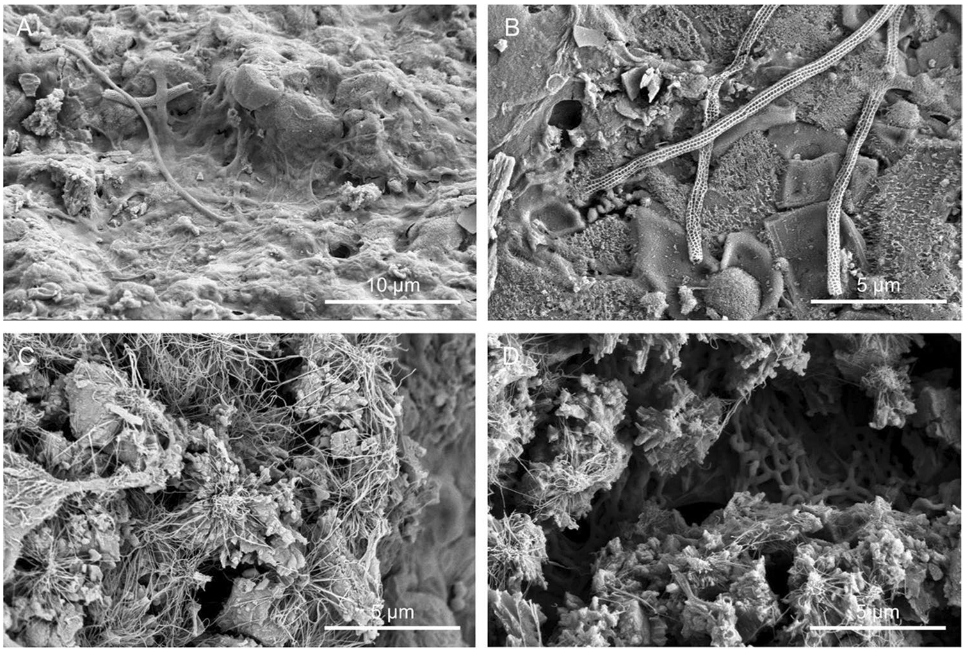


Fig. 5 Field emission scanning electron microscopy images of GL samples. Representative field emission scanning electron microscopy images of the studied samples, depicting **A**, **B** reticulated filaments embedded in a matrix of extracellular polymeric substances (GL_1) and **C**, **D** Actinobacteria-like filamentous forms (GL_3)

Table 1 Statistics. Diversity indexes

Cave name	Touristic use	Samples	Raw reads	Trimmed reads	Observed ASVs	Shannon	Pielou's
Grotta Lunga	High	GL_1	113,047	51,502	1057	8.117803	0.8121241
		GL_3	107,045	48,489	1190	8.762284	0.8618003
Grotta del Santo	High	GS_1	132,755	61,396	621	5.546317	0.6012919
		GS_2A	105,144	21,755	410	5.578653	0.6440540
		GS_2B	128,250	56,174	286	2.527644	0.3113268
		GS_4	132,437	58,838	678	7.348656	0.7855029
Grotta Catanese I	Low	GC1_1A	105,899	43,269	677	5.077743	0.5432766
		GC1_2	124,509	52,204	743	4.797957	0.5055883
Grotta di Monte Corruccio	Low	GMC_1	125,910	55,677	639	4.144329	0.4468860
		GMC_2	108,121	50,496	569	6.867270	0.750339
		GMC_4	141,225	66,672	384	5.293697	0.6199319
		GMC_5	131,544	56,512	405	3.209504	0.3722437

possible relations between the observed parameters of richness and abundance and any environmental factor characterizing the caves we studied. In general terms, major values of richness were observed in "Grotta Lunga" being characterized by low elevation (850 m) and high temperature (18.7 °C). On the contrary, lower values of richness were observed in "Grotta di Monte Corruccio," which is located at 1365 m, with a temperature of 13.9 °C. Tentatively, differences among caves in terms of richness could also be explained by the different environmental conditions and biogeochemical processes operating in caves [56] as well as a possible response of the microbial communities to human disturbance [57]. Accordingly, richness was higher in "Grotta Lunga" and "Grotta Catanese I" experiencing low tourist use. Similar results were obtained by Ikner et al. [58] reporting lower diversity in caves highly exploited for touristic purposes.

Microbial Communities

Independent libraries of DNA sequences from each sample were built targeting the V3-V4 hypervariable regions of the 16S rRNA gene, with the objective of detecting the total bacteria present in these samples.

The relative abundances of the dominant phyla in each cave sample are shown in Fig. 8. Most of the identified bacteria belonged to *Actinomycetota*, *Pseudomonadota*, *Acidobacteriota*, *Chloroflexota*, and *Cyanobacteria*, followed by

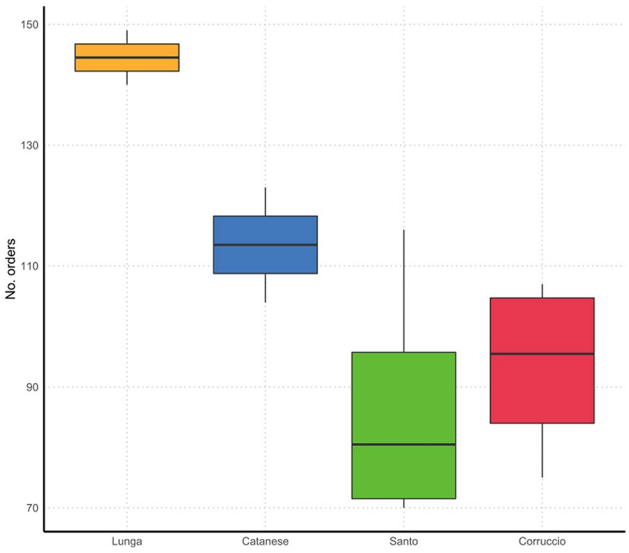


Fig. 6 Number of orders of bacteria observed in the four examined caves

other phyla with lower representativeness. Taxonomic identification resulted in a prominent importance of the phylum *Actinomycetota* in almost all samples, ranging from 78.4% in GS_2B to 14.3% in GC1_2, corroborating the observations by FESEM.

The presence of *Actinomycetota* has been widely found in caves where are actively involved in the biomineralization processes (e.g., Riquelme et al. [3]; Barton et al. [59]). Moreover, members of the phylum *Actinomycetota* represent a promising source of bioactive metabolites for drug development [60, 61].

The phylum *Pseudomonadota* was also present in each cave, being the most representative group in “Grotta del Santo” and “Grotta Lunga” (GS_4, GL_1, and GL_3 with 48.97%, 44.43%, and 42.88%, respectively). The *Pseudomonadota* are well represented in lava caves across the world [62], including New Mexico, Hawaii, Azores, and Galapagos Islands [9, 11, 15].

A similar trend was observed by Gonzalez-Pimentel et al. [8] and Miller et al. [15], where the phyla *Actinomycetota* and *Pseudomonadota* were among the most representative groups in lava tubes from La Palma (Canary Islands) and Galápagos (Equador), respectively.

The phylum *Acidobacteriota* was equally distributed in all caves, ranging from 3.02% in “Grotta di Monte Corruccio” (sample GMC_5) and 12.39% in “Grotta del Santo” (sample GS_2A). Several studies have detected the presence of acidobacterial 16S rRNA gene sequences in caves (e.g., Holmes et al. [63]; Hutchens et al. [64]; Chelius and Moore [65]; Engel et al. [66]). However, their role is still poorly understood [67].

The phylum *Cyanobacteria* was abundant in Grotta Catanese I but poorly represented in the other caves. The presence of this taxon in sample GC1_2 is due to its proximity to the cave entrance, which receives sunlight allowing the development of photosynthetic-based biofilms on the cave wall. Accordingly, *Cyanobacteria* are mainly favored by the presence of light and thereby generally frequent near the cave entrance [68, 69], although the presence of artificial

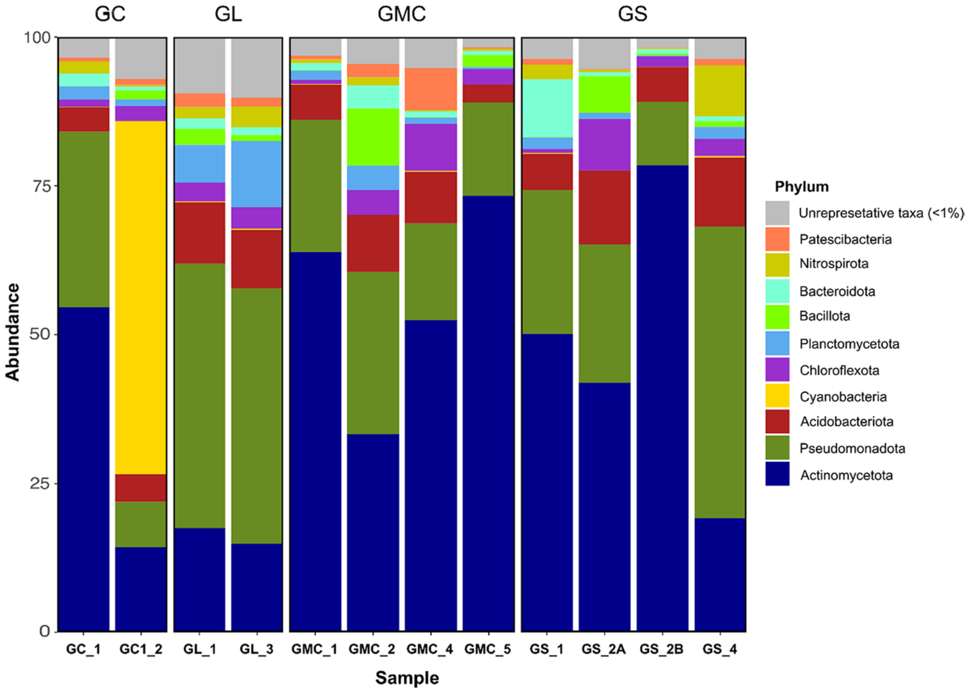
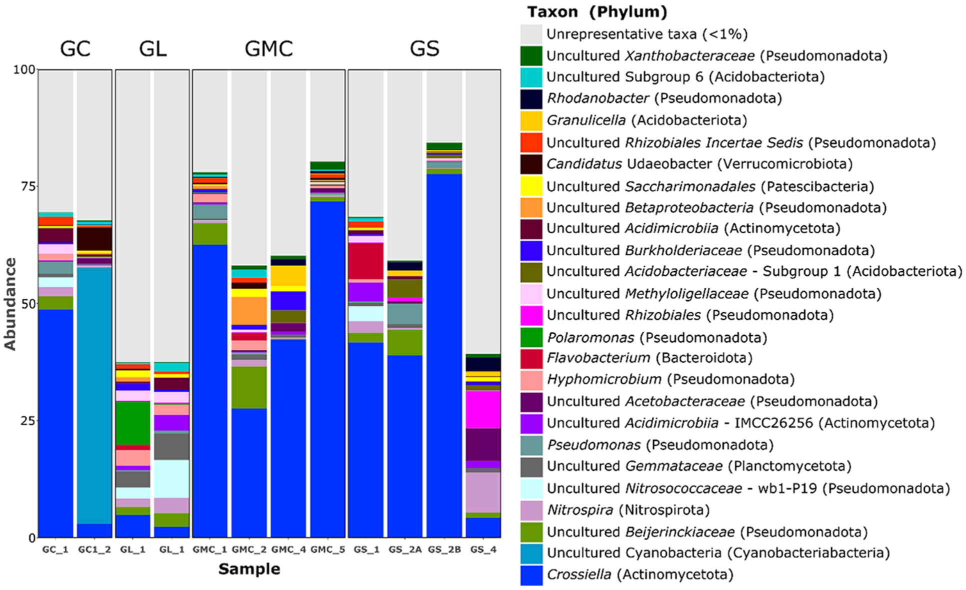


Fig. 7 Distribution patterns of bacterial phyla in the samples (99% ASV cutoff) of “Grotta Catanese I” (GC), “Grotta Lunga” (GL), “Grotta di Monte Corruccio” (GMC), and “Grotta del Santo” (GS)

Fig. 8 Taxonomic compositions of the 12 samples based on the 16S rRNA gene markers (99% ASV cutoff). Identifications reached the genus level with the exception of ASVs identified as uncultured and candidatus bacteria. In brackets (), the affiliation to phylum was included



light can promote their growth in the innermost cave zones (e.g., Piano et al. [70]), which is not the case. Phyla with less representativeness, but with at least 5% of abundance, were *Chloroflexota*, *Planctomycetota*, *Bacillota*, *Nitrospirota*, *Bacteroidota*, *Patescibacteria*, and *Verrucomicrobiota*. They are also common at lower rates in other lava tubes [3, 6, 9]. Other phyla with relative abundances < 1% were also retrieved.

The bacterial communities identified at the class level showed differences between caves and sectors. Most bacteria belonged to the following class: *Actinomycetes*, *Gammaproteobacteria*, *Alphaproteobacteria*, *Oxyphotobacteria*, followed by the less abundant *Bacilli*, *Bacteroidia*, and *Nitrospira* (Fig. 9).

The relative abundance of the different classes varied in each sample, suggesting differences in the local environment and element composition of the volcanic substrate [71]. *Actinomycetes* was the most abundant class in "Grotta del Santo" (GS_2B: 78.21%) and "Grotta di Monte Corruccio" (GMC_5: 72.76%; GMC_1: 62.94%) sample, while *Gammaproteobacteria* was predominant in "Grotta Lunga" (GL_1: 28.60%) and "Grotta del Santo" (GS_4: 25.99%). *Oxyphotobacteria* were instead the most abundant class in "Grotta Catanese I" (GC1_2). The class *Alphaproteobacteria* was also well represented in all samples, ranging from 22.37% in "Grotta del Santo" GS_4 and 4.66% in "Grotta Catanese I" (GC1_2). The class *Actinomycetes* is common in caves and its studies in different locations have revealed the presence of several novels and rare taxon [3, 8, 57]. Investigations on this taxon have highlighted its biotechnological relevance [72–74] as well as their importance as potential pathogens (e.g., Jurado et al. [75]; Gonzalez-Pimentel et al. [8]; Buresova et al. [76]).

The class *Gammaproteobacteria* is among the most dominant group in habitats with either natural or anthropogenic organic inputs [77]. Accordingly, it seems to represent a good bioindicator to detect the presence of contaminants in soil [78–80] or caves [81]. This taxon was abundant in samples GL_1 (28.69%), GL_3 (24.65%) from "Grotta Lunga" and GS_4 (25.99) from "Grotta del Santo," which represent "high tourist use" caves. The presence of pathogens in caves has rarely been discussed and evidence of contamination still results scarce (but see Luong et al. [82]). Among pathogens frequently detected in caves, considered indicators of human impact, there are *Bacillus* spp., *Escherichia coli*, and *Staphylococcus aureus* [81, 83, 84].

The bacterial communities identified at the genus level showed differences between caves and sectors. The genus *Crossiella* is well present in all samples, being the most abundant group in 8 out of 12 analyzed samples (Fig. 8). The taxon was abundant in sample GS_2B from "Grotta del Santo," and GMC_1 and GMC_5 from "Grotta di Monte Corruccio" with a relative abundance respectively of 77.6%, 71.8%, and 62.54%. The genus is common in lava tube caves of Hawaii and Azores [3, 11, 85] and also in limestone caves [86]. Gonzalez-Pimentel [17] observed a high abundance of *Crossiella* in moonmilk from La Palma lava caves. Recent studies have hypothesized the influence of *Crossiella* on the nitrogen cycle and its possible contribution to calcium carbonate precipitation in caves [87, 88].

Some important groups after *Crossiella* were the genus *Pseudomonas*, *Bacillus*, *Chujaibacter*, and *Sphingomonas*.

Nitrospira and the family groups *Beijerinckiaceae* and *Nitrosococcaceae* are respectively described as nitrite-oxidizing, nitrogen fixation, and ammonia-oxidizing bacteria, relatively common in caves [6, 9, 11, 89]. *Chujaibacter* is associated with heavy metal metabolism [90]. The information provided by the identification of these groups of bacteria could respond to the presence of the influence of vegetation and anthropic pressure on the studied caves.

The presence of *Bacillus*, *Pseudomonas*, and *Sphingomonas* in caves is often associated with the presence of high human impact (e.g., Lavoie and Northup [83]; Ikner et al. [58]; Bastian et al. [81]) and recent studies have showed their antimicrobial activity against pathogenic bacteria, as well as a potential source of new microorganism [91, 92]. Other genera identified were as follows: *Alkanibacter*, *Flavobacterium*, *Steroidobacter*, *Sporosarcina*, wb1-P19, *Polaromonas*, this last in clone libraries and culture collections from polar and high-elevation environments [93].

Conclusions

This study provides the first insight into the taxonomic groups constituting the microbial communities of Mount Etna lava tubes. Although the limited number of samples did not allow us to properly correlate these data with any environmental variables, we could detect the higher richness in “Grotta Lunga,” which is located at a lower elevation (850 m a.s.l.), whereas we observed a general lower richness at higher elevation (1365 m a.s.l.) in “Grotta di Monte Corruccio.” Accordingly, differences among caves in terms of richness could be explained by the different environmental conditions and biogeochemical processes operating in caves [55]. Further investigations would be therefore desirable to shed light on which factors drive the richness of microbial communities on Mount Etna lava caves.

Our results revealed the abundant presence of phylotypes previously detected in other lava tubes worldwide. The presence of a large number of unclassified bacteria in the 12 sampling sites suggests that these lava tubes have a great potential for the isolation of novel species. However, the land use overlying the cave, as well as the uncontrolled presence of visitors into these caves, may pose major threats to these ecosystems, especially those located at lower altitudes beyond the strictly protected areas of the volcano, generally exposed to a higher tourist frequentation. Further research is needed to ascertain the possible presence of novel bacteria in caves from the Etna lava caves, as well as the presence of contaminants and their potential risk associated with human health. Data of pathogens should be therefore considered not only for the conservation of these unique geosites, but also for the risk associated with human health.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-023-02181-2.

Acknowledgements We thank the “Ente Parco dell’Etna” that authorized and allowed the carrying out of the scientific research activity in the Park.

Author Contribution GN and AZM conceived the study and performed the sampling activities. Funding was secured by AZM. Laboratory protocols were conducted by GN and AZM. JGP, EP, AZM, and GN analyzed the data. The first draft of the manuscript was written by GN along with significant contributions from JGP, EP, MI, and AZM. AZM and MI contributed to the review and editing of this manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. This work was supported by the Spanish Ministry of Science and Innovation (MCIN) under the research project TUBOLAN PID2019-108672RJ-I00 funded by MCIN/AEI/10.13039/501100011033. In addition, this work received support from the Portuguese Foundation for Science and Technology (FCT) under the MICROCENO project (PTDC/CTA-AMB/0608/2020), and from the Spanish National Research Council (CSIC) through the intramural project PIE_20214AT021. A.Z.M. was supported by the CEECIND/01147/2017 contract from FCT and the Ramón y Cajal contract (RYC2019-026885-I) from the MCIN.

Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

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Paper IV

International Journal of
Environmental Research and Public Health

Article

Sulfidic Habitats in the Gypsum Karst System of Monte Conca (Italy) Host a Chemoautotrophically Supported Invertebrate Community

Giuseppe Nicolosi [1,2,*], Sandro Galdenzi [3], Maria Anna Messina [2], Ana Z. Miller [4,5], Salvatore Petralia [2,6], Serban M. Sarbu [7] and Marco Isaia [1]

[1] Department of Life Sciences and Systems Biology, University of Turin, 10124 Torino, Italy; marco.isaia@unito.it
[2] Centro Speleologico Etneo, 95123 Catania, Italy; messina.marianna@gmail.com (M.A.M.); salvatore.petralia@unict.it (S.P.)
[3] Scuola di Scienze e Tecnologie—Sezione di Geologia, Università di Camerino, 62032 Camerino, Italy; galdenzi.sandro@tiscali.it
[4] Instituto de Recursos Naturales y Agrobiología de Sevilla, IRNAS-CSIC, 41012 Sevilla, Spain; anamiller@irnas.csic.es
[5] HERCULES Laboratory, University of Évora, 7000-809 Evora, Portugal
[6] Dipartimento di Scienze del Farmaco e della Salute, University of Catania, 95124 Catania, Italy
[7] Emil Racoviţă Institute of Speleology, Str. Frumoasa nr. 31, 010986 Bucureşti, Romania; serban.sarbu@yahoo.com
* Correspondence: giuseppe.nicolosi@unito.it

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Citation: Nicolosi, G.; Galdenzi, S.; Messina, M.A.; Miller, A.Z.; Petralia, S.; Sarbu, S.M.; Isaia, M. Sulfidic Habitats in the Gypsum Karst System of Monte Conca (Italy) Host a Chemoautotrophically Supported Invertebrate Community. *Int. J. Environ. Res. Public Health* **2022**, *19*, 2671. https://doi.org/10.3390/ijerph19052671

Academic Editor: Paul B. Tchounwou

Received: 17 December 2021
Accepted: 23 February 2022
Published: 25 February 2022

Abstract: The great diversity of the invertebrate community thriving in the deepest sections of the gypsum karst system of the Monte Conca sinkhole (Sicily, Italy) suggests the existence of a complex food web associated with a sulfidic pool and chemoautotrophic microbial activity. To shed light on the peculiarity of this biological assemblage, we investigated the species composition of the invertebrate community and surveyed trophic interactions by stable isotope analysis. The faunal investigation conducted by visual censuses and hand sampling methods led to the discovery of a structured biological assemblage composed of both subterranean specialized and non-specialized species, encompassing all trophic levels. The community was remarkably diverse in the sulfidic habitat and differed from other non-sulfidic habitats within the cave in terms of stable isotope ratios. This pattern suggests the presence of a significant chemoautotrophic support by the microbial communities to the local food web, especially during the dry season when the organic input from the surface is minimal. However, when large volumes of water enter the cave due to local agricultural activities (i.e., irrigation) or extreme precipitation events, the sulfidic habitat of the cave is flooded, inhibiting the local autotrophic production and threatening the conservation of the entire ecosystem.

Keywords: gypsum cave; stable isotope ecology; food web analysis; cave-dwelling fauna; sulfide; ecosystem conservation

1. Introduction

In terms of energy inputs, subterranean ecosystems are generally regarded as oligotrophic environments, especially in the inner and most isolated parts [1]. Nutrient supplies mainly depend on the flux of energy from the surface, which enters the subterranean habitats in a variety of ways: carried by gravity from the cave entrances and ceiling cracks, by water, wind, or by animals such as bats, birds or rodents [1–6].

Several studies have underlined how some subterranean ecosystems do not depend entirely on organic matter coming from the surface [7–13] but instead, on chemoautotrophic in situ production of organic matter by microorganisms processing different inorganic elements to obtain energy and nutrients. These organisms represent de facto the only possible primary producers inhabiting the subterranean ecosystem. In some cases, such non-photoautotrophic subterranean biological assemblages support unusually rich local biological communities, even when they are completely isolated from the surface [14–17]. Moville Cave in Romania represents one of the most interesting and well known examples of a chemoautotrophic hypogean system, characterized by hydrogen sulfide-rich groundwater [18]. As demonstrated by carbon and nitrogen stable isotope analyses [7], the cave is a closed system, fully supported by chemosynthesis. Here Vlasceanu et al. [19] reported the presence of *Thiobacillus thioparus*, a bacterium capable of oxidizing hydrogen sulfide to sulfuric acid. The organic material produced in situ allows the development of a rich and diversified community composed of different species, 37 of which are endemic of this subterranean groundwater ecosystem [13,18,20,21]. In other cases, chemoautotrophic bacterial activity represents an additional—but not exclusive—energy source in the cave. This is the case of semi-closed hypogean systems such as the Cesspool Cave in USA [16,22].

In this paper, we aimed to describe the biological community dwelling in the Monte Conca sinkhole (W-Sicily, Italy) and to demonstrate the role of the resident chemoautotrophic organisms in the cave food web, providing evidence for their independence from external inputs. For these purposes, we identified the most abundant and detectable species dwelling in the cave, we sorted them in two groups according to their occurrence in sulfidic and non-sulfidic habitats, and we compared the relative abundance of C and N stable isotopes in the two groups by means of Stable Isotope Ratio Analysis (SIRA).

2. Materials and Methods

2.1. Site Description

The Monte Conca sinkhole (cadastral number 3000SI-CL, 37°29′19.7′′ N 13°42′46.5′′ E) is an active cave developed in Messinian evaporites occurring in central western Sicily (Italy), within the Strict Nature Reserve of "Monte Conca". It is also a Geosite of regional interest, only accessible based on authorized permission. Currently, Monte Conca sinkhole is considered the longest and deepest gypsum karst system in Sicily, reaching a depth of 130 m and a total passage development of more than 2.5 km [23]. The entrance gallery is about 100 m long, followed by four shafts of 11, 12, 35 and 26 m deep, respectively (Figure 1a). At the bottom of the fourth shaft, a 450 m long gallery leads to the terminal part of the cave, where a sulfidic pool is located, fed by a stream seeping from bedrock (Figure 1b). Physical and chemical analyses conducted by previous researchers [24] have provided robust evidence for the presence of bacterial activity, as witnessed by filamentous microbial mats floating on the water surface of the sulfidic waters (Figure 1c) and by organic stalactites (snottites) on the cave walls and ceilings (Figure 1d), rich in sulfuric acid (H_2SO_4). Davis et al. [25] documented the presence of the microbial community dominated by sulfur-oxidizing bacteria.

The cave floor is predominantly bedrock, frequently covered by the stream. Sediments of different origin such as clay, mud and gravel and chemical deposits (iron and gypsum deposits), are visible in the lower parts of the gallery.

During the wet season—generally from January until May—large volumes of water enter the cave from a tributary of the Gallodoro stream, flooding it entirely [24] and mostly precluding access to the cave. Such floods result in massive inputs of allochthonous surface materials into the cave as testified by remarkable accumulations of mud and organic matter in the lower galleries that may even hamper speleological progression. Surface organic debris also percolate through the fractured ceiling, providing an additional source of external organic carbon. Water derived from surface-runoff during the wet season also facilitates the entry of anthropogenic microbes, including potential contaminants, such as *Escherichia* and *Lysinibacillus* bacteria which are likely derived from outside, particularly from the agricultural fields located above the cave [25].

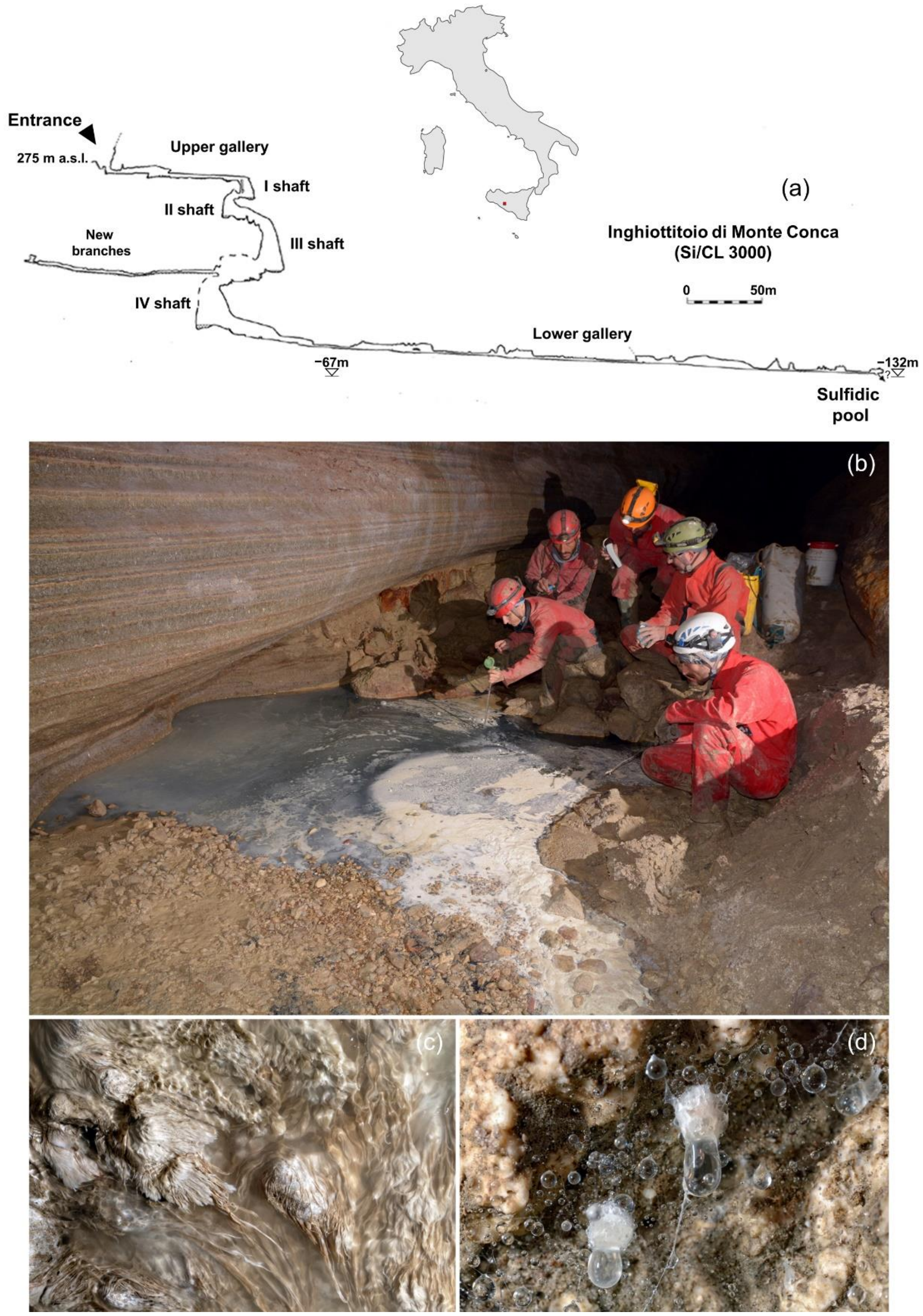

Figure 1. (**a**) Map of the study area (topography of the Monte Conca sinkhole (Adapted with permission from Madonia and Vattano [23]. 2008 Vattano). (**b**) Sulfidic habitat at the end of the lower gallery containing sulfur suspensions on water surface. (**c**) Filamentous microbial mats floating on the sulfide pool surface. (**d**) Acid droplets (snottites) hanging from the ceiling of the cave. Photos: F. Fiorenza.

In July and August, i.e., the dry season, the stream dries out and no water flows inside the cave, leaving only small and isolated water pools. According to recent microbiological studies [25], such conditions primary favor sulfur-oxidizing bacteria such as *Sulfurovum*, *Sulfurimonas*, *Thiovirga* and *Arcobacter*. However, sudden extreme meteorological events during summer may fill up the cave altering these peculiar conditions, besides shutting the way out to speleologists and making investigations in the cave particularly hazardous.

2.2. Habitat Characterization

Habitats along the cave were characterized by means of chemical (pH and sulfide concentration) and physical (water and air temperature) parameters. The measurements were performed in a non-sulfidic control pool located under the fourth shaft (non-sulfidic habitat, blue circle in Figure 2a) and in a pool in the terminal gallery (sulfidic habitat, yellow circle in Figure 2a).

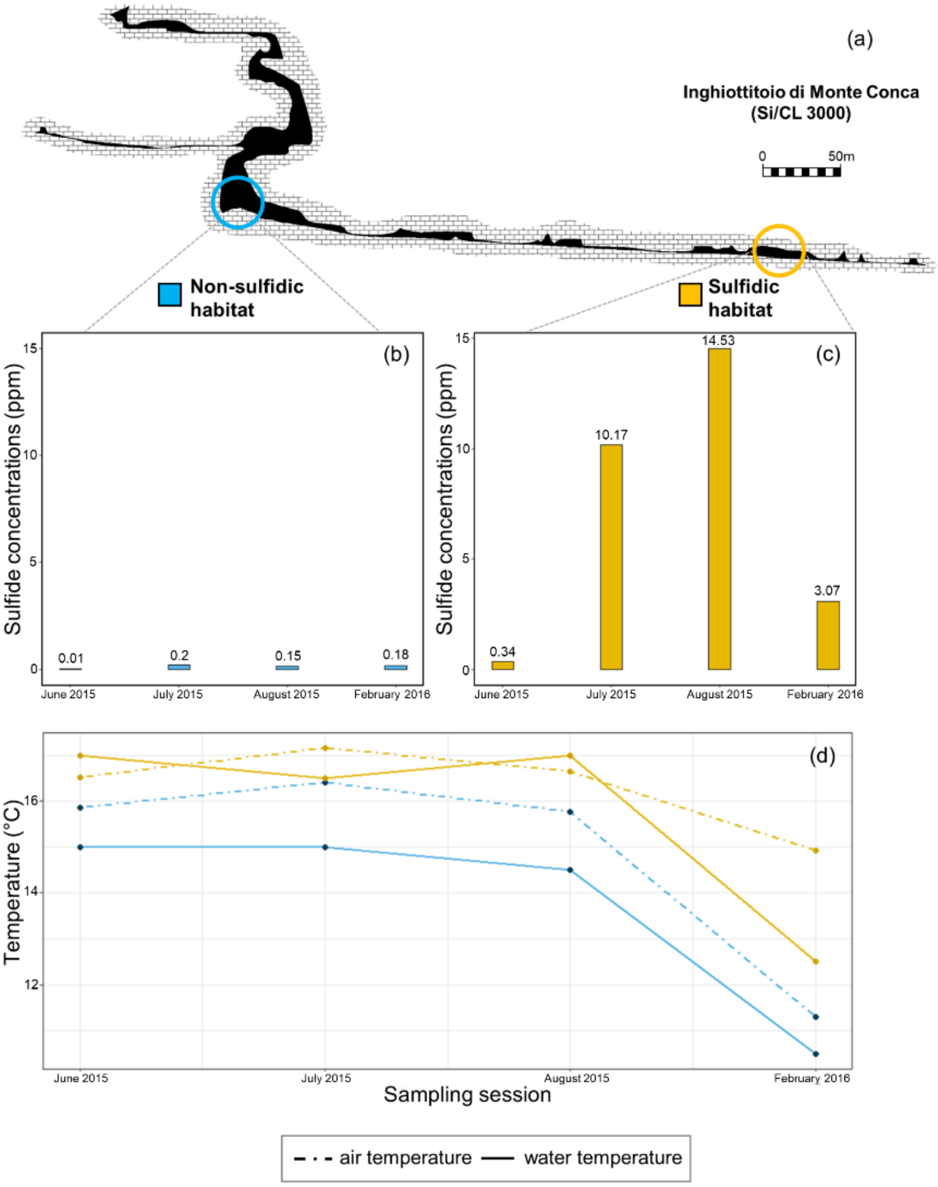

Figure 2. (**a**) Map of the study area with the position (circles) of the sampling sites: blue (non-sulfidic habitat) and orange (sulfidic habitat). Topography of the Monte Conca sinkhole (Adapted with permission from Madonia and Vattano [23]. 2008 Vattano). (**b**) Sulfide concentrations (ppm) in the non-sulfidic and sulfidic (**c**) habitats monitored across four sampling sessions. (**d**) Air and water temperature in Monte Conca sinkhole recorded during the sampling session (June, July, August 2015, and February 2016) at non-sulfidic and sulfidic habitats.

The sulfide concentration in the water was measured by Cline's methylene blue method [26]. For this purpose, 10 mL of water for each sample were stabilized with 1.5 mL of Zn acetate. The solution was added with a volume of 10 mL of N,N, dimethyl–p-phenylendiammonium solution and 1 mL of iron(III) chloride solution. Once in the laboratory, after 30 min of stirring at room temperature, the absorbance value at 666 nm was measured for each sample by a Molecular Devices SpectraMax® spectrophotometer. The amount of sulfide was then calculated using calibration lines in the range from 0.1 to 20 ppm. Sulfide concentration was measured at the two sampling sites (control and sulfidic pools) in four sampling sessions (June, July, August 2015, and February 2016).

Air and water temperature were measured by using a HOBOware sensor (sensitivity, 0.01 °C) and a CM-35 Crison multimeter probe respectively. Measurements were taken during each of the four sampling sessions in both the sulfidic and control pools.

2.3. Biological Survey

To obtain an accurate knowledge of the biological community dwelling in the cave, we conducted four surveys, two in the dry and two in the wet season (Table 1). The high level of risk associated with the access and the permanence in the cave precluded us from using standardized sampling methodologies (i.e., pitfall trapping) that would allow us to examine abundance trends in invertebrates. Accordingly, both the aquatic and terrestrial community were investigated in terms of presence/absence data.

Table 1. Calendar of the sampling sessions and meteorological data provided by SIAS (*Servizio Informativo Agrometeorologico Siciliano*, Italy).

Date	Season	Rainfall (mm) of the Previous Month	Number of Rainy Days in the Previous Month	Daily Mean Outside Temperature
20 June 2015	wet	44.2	8	20.28
11 July 2015	dry	0	0	26.16
29 August 2015	dry	18	5	25.5
6 February 2016	wet	117	21	7.55

Species were monitored through visual census, although identification often required the collection of specimens.

Terrestrial invertebrates were collected manually using tweezers, searching walls, floor, ceiling, and turning over rocks and debris. Aquatic macroinvertebrates were collected using a needle-less syringe (60 mL) in the pools and a fine mesh net (60 micron) along the stream. Additionally, we used bottle traps to collect amphipods.

All specimens were sorted under a stereomicroscope and identified to the lowest possible taxonomic level. Material was preserved in 70% ethanol. For certain groups requiring DNA analysis (i.e., Clitellata), we preserved specimens in 95% ethanol.

Identifications were supported by specialists (Gastropoda, Clitellata, Copepoda, Amphipoda) (see Acknowledgements). A number of specimens (Ostracoda) could not be identified to the species level. Nomenclature for all groups follows the Global Biodiversity Information Facility database [27].

2.4. Stable Isotope Analysis

We used Stable Isotope Ratio Analysis (SIRA) of carbon and nitrogen to determine the level of independence from the surface of the food-web as suggested by Sarbu et al. [7] (see also De Niro and Epstein [28], Vlasceanu et al. [29], Engel et al. [30], Paoletti et al. [31] and Michener and Lajtha [32]. SIRA represents an effective tool to determine food sources in a given ecosystem because organisms fractionate isotopes of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{13}N$) in predictable ways. For this purpose, we only focused on the organisms collected in the dry season i.e., when maximal isolation from the surface is achieved. Samples were assigned to the "sulfidic habitat" category when collected within or in close proximity to the sulfidic pools, and to the "non-sulfidic habitat" when collected within, or in close proximity to, the control pool (Figure 2a).

Analyses were performed on the most detectable species. Samples were placed in falcon tubes and, once in laboratory, washed with deionized water and dried. Samples of *Nepa cinerea* were also brushed to remove the bacterial coated layer formed within the sulfidic pool. Samples of *Tubifex blanchardi* and *Proasellus montalentii* were composed of several individuals to guarantee sample size for mass spectrometry. Large macroinvertebrates were analyzed individually.

Moreover, we collected a sample of the white biofilm in the sulfidic pool as this could represent the food source for a chemoautotrophically-based ecosystem. For comparison, a sample of organic matter was also collected in the non-sulfidic habitat (i.e., decaying plant and other organic remains of surface origin).

Isotopic composition was determined using an isotope mass spectrometer. Stable isotope data are presented in the delta (δ) notation as the relative difference between the ratios of the sample and the standards: $\delta^{13}C = [(^{13}C/^{12}C) - 1] \times 1000$ and $\delta^{15}N = [(^{15}N/^{14}N) - 1] \times 1000$, where $\delta^{13}C$ or $\delta^{15}N$ are reported in part per thousand (‰). Atmospheric N_2 is the standard for nitrogen, while Vienna PeeDee belemnite (VPDB) is the standard for carbon.

The Stable Isotope Ratio Analysis was performed at the University of New Mexico, Department of Earth and Planetary Sciences, Albuquerque, NW, USA.

To test for the actual separation between the sulfidic and non-sulfidic habitats, $\delta^{13}C$ values were statistically compared with the Student's *t*-test. For this purpose, only the species shared by the two habitats were analyzed. Normality was at first tested using the 'shapiro.test' function from 'stats' package, ver. 4.2.0 [33]. The F-test was used to check for homogeneity in variances by using the 'var.test' function from 'stats' package. The main test was performed using the 't.test' function from 'stats' package.

3. Results

3.1. Habitat Characterization

Sulfide concentrations were negligible (0.03 ppm and 0.18 ppm) in the control plot (Figure 2b). Values of H_2S in the sulfidic habitat (Figure 2c) were generally higher, ranging from 0.34 ppm (June) to 14.53 ppm (August).

Air and water temperatures in the control site were relatively stable in June, July and August (around 16 °C and 15 °C, respectively). Temperature dropped in February, reaching 11.5 °C and 10.5 °C, respectively. (Figure 2d).

Air and water temperature in the sulfidic habitats followed the same trend across the year, but values were generally higher than those measured in the non-sulfidic habitat, with values at least 0.6 °C higher (June). The highest deviation was +3.6 °C, recorded in February (Figure 2d). Water temperature differed between the sulfidic and non-sulfidic habitats, with +1.5 °C in July 2015 and +2.5 °C in August (Figure 2d).

3.2. Biological Survey

The biological survey determined the presence of 54 species (Table 2, see also Supplementary Material (Table S1) for the complete species list), including 27 species collected at the cave entrance, 39 in the non-sulfidic habitat and 48 in the sulfidic one. However, just a few of them showed subterranean adaptations, thus demonstrating a general epigean origin of the invertebrate assemblage colonizing the cave. Some of the species, such us the water scorpion *Nepa cinerea* or the beetle *Paranchus albipes* were found in the dry and the wet season, hinting at their possible presence in the cave throughout the year. The general epigean origin of the assemblage is related to passive water transportation during the wet season, contributing to an overall and seasonal increase of the species diversity at the inner parts of the cave. Predators were highly diversified in the assemblage (24 species), including 18 species at the cave entrance, 16 in the non-sulfidic habitat and 20 in the sulfidic one. Detritivores were represented by 20 species, (16 at the cave entrance, 20 in the non-sulfidic habitat and 20 in the sulfidic one).

Table 2. Number of invertebrate species sorted in trophic groups (predators, detritivores, and others i.e., omnivores, herbivores or phytophagous) collected in the three sectors of the Monte Conca sinkhole during the wet (W) and the dry (D) season.

Cave Sector	Predators		Detritivores		Others		Total	
	W	D	W	D	W	D	W	D
Entrance	18	9	3	3	6	4	27	16
Non-sulfidic	16	7	16	7	7	5	39	19
Sulfidic	20	9	20	14	8	6	48	29
Total	24	20	20	18	9	8	54	43

During the dry season, the lack of surface water flowing in the cave reduces the intake of epigean species, drastically reducing the overall number of species in the cave. Predators drop dramatically in both sulfidic (9) and non-sulfidic habitats (7), as well as the number of detritivores (14 and 7 species respectively), that become the most diverse group in the sulfidic habitat (Table 2).

The aquatic invertebrate community was remarkably diverse, especially in the sulfidic habitat where we detected the presence of numerous species in all trophic levels, including detritivores such as *Pseudamnicola (Pseudamnicola) moussonii* (Gastropoda, Hydrobiidae), *Proasellus montalentii* (Malacostraca, Asellidae) and *Tubifex blanchardi* (Clitellata, Naididae). Among predators, we detected *Haemopis sanguisuga* (Clitellata, Haemopidae) and *Nepa cinerea* (Insecta, Nepidae) (Figure 3a). The latter was also present in the control habitat, but in lower numbers.

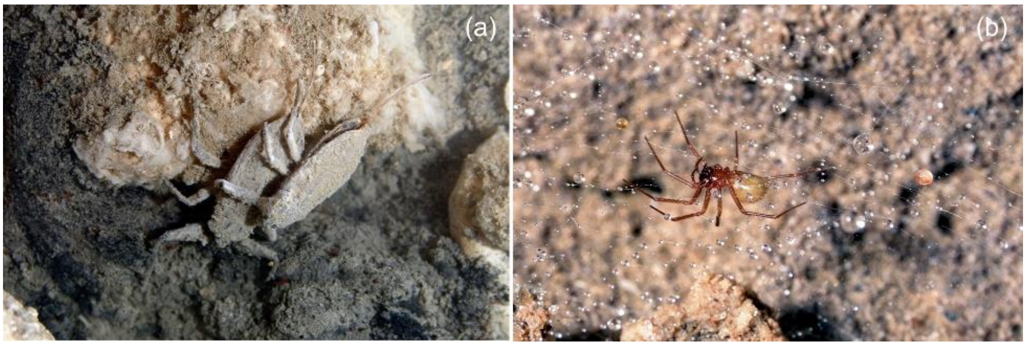

Figure 3. (**a**) The water scorpion *Nepa cinerea* dwelling in the sulfidic waters. (**b**) The spider *Lessertia barbara* on its webs with droplets of sulfuric acid (pH ~1). Photos: F. Fiorenza.

Among terrestrial species, spiders were usually found under rocks, dead wood or in crevices in the walls. Some were only present in the twilight zone of the cave (e.g., *Metellina merianae*, *Holocnemus pluchei*), while others, such as the troglophile *Kryptonesticus eremita*, were also found dwelling on organic debris and on cave walls in the terminal section of the cave, in both sulfidic and non-sulfidic habitats.

The spider *Lessertia barbara* was spotted dwelling on some heaps of organic material in proximity of the sulfidic pools (Figure 3b). Here, we noticed the presence of the spider egg sacs hanging from the spider webs among droplets of sulfuric acid (pH ~1) sticking on the web threads (Figure 3b).

3.3. Stable Isotope Analysis

Stable isotope analysis was performed on the most detectable biological species. For the sulfidic habitat we analyzed n = 3 *Agabus* sp. (Insecta, Coleoptera), n = 1 larva and 1 adult of *Meladema coriacea* (Insecta, Coleoptera), n = 4 *Kryptonesticus eremita* (Arachnida, Araneae), n = 3 *Nepa cinerea* (Insecta, Hemiptera), n = 3 *Paranchus albipes* (Insecta, Coleoptera), n = 1 sample of *Proasellus montalentii* (Malacostraca, Asellidae), n = 1 sample of *Tubifex blanchardi* (Clitellata, Haplotaxida). For the non-sulfidic habitat, we analyzed n = 1 larva Dytiscidae

(Insecta, Coleoptera), *n* = 1 Julidae (Diplopoda), *n* = 5 *Kryptonesticus eremita*, *n* = 1 *Meladema coriacea*, *n* = 2 *Nepa cinerea*, *n* = 1 *Oxychilus lagrecai* (Gastropoda, Stylommatophora).

The results of isotope analysis revealed the presence of an autochthonous food source. In particular, the aquatic biofilm collected in the sulfidic habitat (presumably sulfur-oxidizing microorganisms) was isotopically light, showing a δ^{13}C value of −40.18‰ and a δ^{15}N value of 11.77‰, indicating a chemoautotrophic food source. This differed significantly from the organic matter collected in the control habitat, where values of δ^{13}C were −23.46‰ (Figure 4a), indicating differential use of carbon sources. The C:N ratio for the white biofilm was 4.8, in accordance with white filament bundles observed in Lower Kane Cave (C:N ratios ~5), suggesting a high-quality food source [16]. Contrarily, the C:N value for the organic matter in the control pond was higher, reaching 8.2–13 attesting the lower quality of the food source.

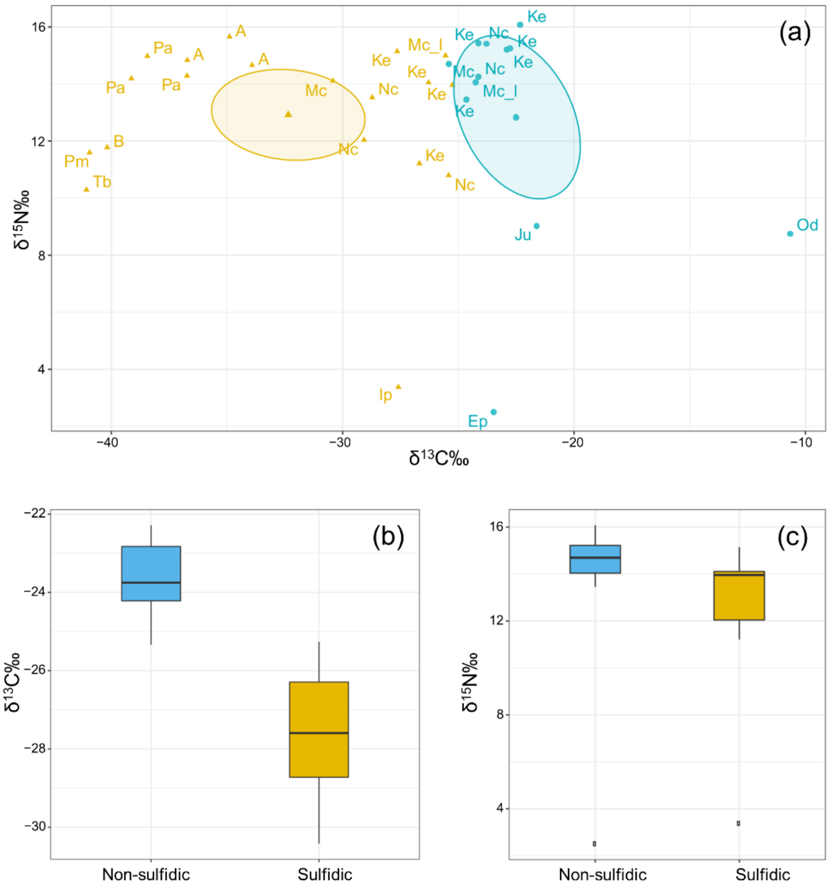

Figure 4. (**a**) Scatter plot of δ^{15}N against δ^{13}C in sulfidic (yellow) and non-sulfidic (blue) habitat. The samples differ in their δ^{13}C, indicating differential use of carbon sources. Abbreviations: A = *Agabus* sp., B = biofilm, Oc = organic matter collected in the control pool (non-sulfidic habitat), Ju = Julidae, Ke = *Kryptonesticus eremita*, Nc = *Nepa cinerea*, Mc = *Meladema coriacea*, Mc_l = *Meladema coriacea* (larvae), Od = *Oxychilus lagrecai*, Pa = *Paranchus albipes*, Pm = *Proasellus montalentii*, Tb = *Tubifex blanchardi*. (**b**) Boxplot of the δ^{13}C‰ values referred to sulfidic vs. non-sulfidic habitat. (**c**) Boxplot of the δ^{15}N‰ values referred to sulfidic vs. non-sulfidic habitat.

Samples collected in the sulfidic habitat were isotopically lighter in carbon (δ^{13}C = −41.08 to −25.26‰) but rather similar in nitrogen (δ^{15}N = 10.29 to 15.66‰) than the one collected in the non-sulfidic habitat (Figure 4a). The latter were isotopically heavier in carbon (δ^{13}C = −10.64 to −25.34‰) but similar in nitrogen (δ^{15}N = 8.75 to 16.08‰). For instance, the δ^{13}C value for *Tubifex blanchardi* and *Proasellus montalentii* were −41.078‰ and −40.94‰, respectively. Both *T. blanchardi* and *P. montalentii* likely feed on microbial mats as their δ^{13}C values differ considerably from the other invertebrates collected in the sulfidic habitat (Figure 4a). This interpretation is consistent with Rodriguez et al. [34] having documented for *T. blanchardi* the use of bacteria as a food resource.

The beetles *Agabus* sp. and *Paranchus albipes* showed isotopically lighter values of δ^{13}C (−35.17 and −30.10‰, respectively). As both are predators, they probably feed on organisms isotopically lighter such as the isopod *Proasellus montalentii*.

Species present in both the sulfidic and the control habitat, such as the bug *Nepa cinerea*, the spider *Kryptonesticus eremita* and the beetle *Meladema coriacea*, allowed a direct comparison of their isotopic values and C:N ratios. For these specimens, the δ^{13}C values were also isotopically heavier in the control habitat (respectively −23.93, −23.29, −25.33‰) compared to the sulfidic one (−27.74, 26.47, −30.42‰). The C:N ratio values were similar in both habitats. Predators in the non-sulfidic habitat *Nepa cinerea, Kryptonesticus eremita* and *Meladema coriacea* had a value of 4.2, 4.1 and 3.9, respectively, whereas in the sulfidic habitat, their values were 4.2, 4.3 and 4.3, respectively.

Differences among the two habitats in terms of δ^{13}C values were statistically significant (t = −5.75, df = 16, *p*-value =< 0.001) confirming a clear separation of the two habitats, at least during the dry period (Figure 4b). Values of δ^{15}N were comparable in the two habitats (Figure 4c).

4. Discussion

The Monte Conca sinkhole is a gypsum karst system containing a sulfidic pool with documented microbial chemoautotrophic activity [24].

The number of subterranean invertebrate species in Monte Conca sinkhole is rather low if compared with other caves with similar chemical-physical characteristics, such as the Frasassi caves in Italy and Movile Cave in Romania [13,35].

The intermittent action of the stream favors the accidental introduction of surface-dwelling invertebrates in the cave during the wet season. Consequently, surface organisms that are transported in the lower galleries of the cave can temporarily colonize the terminal section of the cave. In contrast, during the dry season their numbers decrease drastically, nearly disappearing.

On the one hand, the presence of a dry season prevents the entrance of accidental species, yet on the other, it seems to favor the resident cave fauna, especially those organisms dwelling in the sulfidic habitat. Our results suggest that the existence of a non-photosynthetic food source sustains a high biodiversity in the cave, especially in the sulfidic ponds. The great diversity of predators and omnivores suggests the presence of a more complex trophic web compared to non-sulfidic habitats within the cave, where the nutrient supplies likely have an allochthonous (epigean) origin. Remarkably, the number of species of detritivores in the sulfidic habitat was twice as high as in the control habitat. It seems likely that such diversity is favored by the high quantity and quality of the white autotrophic microbial biofilm representing the base of the food web in the sulfidic habitat.

During the dry season, dense clusters of *T. blanchardi* dwell in the H_2S-rich water of the sulfidic pool. Here, they probably proliferate facilitated by their ability to survive for long periods in anoxic, sulfidic or heavily polluted areas (e.g., Volpers and Neumann [36] and Martins et al. [37]) as well as by the great availability of bacteria as food resource [34]. The great availability of prey (i.e., *Proasellus montalentii* and *T. blanchardi*) in the sulfidic pools also parallels the presence of a diversified assemblage of predators. Among others, the water-scorpion *Nepa cinerea* can tolerate moderate levels of pollution [38] and so to thrive in sulfidic waters, as previously documented by several authors [39–42].

The spider *Lessertia barbara* does not appear to be at disadvantage by the extreme conditions characterizing the sulfidic habitat. The species was previously uniquely known in Italy from another gypsum cave in Sicily (“Grotta dei Panni”, Santa Ninfa, Trapani), [43]. Remarkably, the species has been described on material collected in a cave in Algeria (“Grotte du lac souterrain” near the Hammam Maskhoutine springs [44]), in several caves in southern Spain [45], and one cave in Morocco [46]. Interestingly, all of these caves are characterized by the presence of sulfidic waters.

The occurrence of nesticid and linyphiid spiders (*Kryptonesticus* spp. and *Phanetta subterranea* Emerton 1875) living close to the sulfidic pools and acid droplet on the spider webs were also observed in Movile, Frasassi and Parker caves [16,47].

According to our results, it seems likely that the proliferation and greater richness of the resident fauna in the terminal part of the cave is favored by the high sulfide concentration characterizing cave waters during the dry season. As suggested by Davis et al. [25], in Monte Conca sinkhole the higher concentration of sulfide favors the prevalence of primary sulfur oxidizers in the microbial community, such as *Sulfurovum*, *Sulfurimonas*, *Thiovirga*, and *Arcobacter*, causing the increase in the production of organic carbon chemosynthetically.

The literature describes elaborate food webs of macroinvertebrates based on chemoautotrophic production in several subterranean ecosystems [7,12,13,34,48] and deep-sea hydrothermal vents and seeps [49,50].

In Monte Conca sinkhole, invertebrates can graze on the in situ produced biomass. In fact, the carbon isotope composition of fauna samples showed markedly lighter values from the sulfidic habitat. This was especially true for organisms feeding directly on bacteria and sediments, such as *Proasellus montalentii* and *Tubifex blanchardi*. The same trend was observed in other sulfidic caves where grazers showed δ^{13}C values consistent with consumption of the microbial mats (e.g., Sarbu et al. [35] and Galdenzi et al. [51]).

The isotopic values for invertebrates that live in the sulfidic habitat differed significantly from those collected in the non-sulfidic one. Similarly, Roach et al. [52] revealed that most of the carbon and nitrogen obtained by fish from the sulfidic Cueva del Azufre stream derived from in situ chemoautotrophic production, whereas detritus and green algae were the dominant food sources of surface-dwelling fish from non-sulfidic stream habitat.

However, besides the driest period, the flooding during the wet season prevents the establishment of trophic chains exclusively based on chemoautotrophic production. The wet season causes large volumes of water to runoff from the surface and enter the Monte Conca sinkhole. This input facilitates the contact between the sulfidic and non-sulfidic habitats and a strong dilution of the hydrogen sulfide content of the water. The sulfide concentration measured was in fact considerably reduced in the wet season, also in the sulfidic habitat.

Davis et al. [25] detected a major abundance of microorganisms derived from anthropogenic inputs during the wet season, rather than sulfur oxidizing organisms. They included the bacteria *Escherichia* and *Lysinibacillus*, likely derived from the agricultural regions around the Monte Conca sinkhole. As reported by several authors, changes in surface land use, such as agriculture activities or sewerage system in urban areas, might alter the amount and composition of water entering the cave, promoting changes in speleothem growth and in cave microbial diversity [53–55]). González-Pimentel et al. [56] revealed a significant input of anthropized agricultural material from the overlying layers into siliceous speleothems from a lava tube in La Palma Island (Spain), inducing changes on the subsurface microbial diversity. In the Monte Conca sinkhole, although included in the Strict Nature Reserve "Monte Conca", the presence of agricultural activities poses a threat to this ecosystem, favoring the input of contaminants of anthropogenic origin and altering the natural hydrological regime of the sinkhole. Recent observations have highlighted a constant input of water even during the summer period, probably derived from the irrigation system upstream of the cave entrance. Besides, climatic changes i.e., extremization of meteorological events such as sudden violent summer rains and cloudbursts, are likely to have a similar impact on the natural hydrological regime, inhibiting chemoautotrophic productivity due to water dilution.

5. Conclusions

Monte Conca sinkhole is a karst system developed in gypsum. It represents one of the few spots in the world that includes a sulfidic pool located within the cave, where the subterranean invertebrate community relies seasonally on autochthonous food produced chemoautotrophically by sulfur-oxidizing bacteria. Accordingly, the Monte Conca sinkhole could be considered an intermediate semi-closed ecosystem, unlike fully closed systems such as the Movile Cave in Romania, and open ones, as found in most sulfidic caves across the world [16,22].

The land use overlying the cave, in particular agricultural activities and extremization of meteorological events pose major threats to this ecosystem, as changes in the natural hydrological regime might increase the amount of water flowing into the cave, favoring the input of anthropogenic pollutants and altering the structure of its unique chemotrophic based food chain. Consequently, the amount of organic matter produced by the sulfur oxidizers, crucial for the structuring of the local biological assemblage, could fade over time. Such alterations could lead to severe disturbance of this exceptional ecosystem, thus threatening its existence. Due to its considerable scientific importance, Monte Conca sinkhole needs immediate and unquestionable protection.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijerph19052671/s1, Table S1: List of taxa collected within the Monte Conca sinkhole.

Author Contributions: Conceptualization, G.N., S.M.S. and M.I.; methodology, G.N., S.G., A.Z.M., S.M.S., M.A.M., S.P. and M.I.; validation, G.N., S.G., A.Z.M., S.M.S., M.A.M., S.P. and M.I.; formal analysis, G.N.; investigation, G.N., M.A.M. and S.P.; data curation, G.N.; writing—original draft preparation, G.N.; writing—review and editing, S.G., A.Z.M., S.M.S., M.A.M., S.P. and M.I.; visualization, G.N.; supervision, M.I. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding. A.Z.M. is supported by a Ramón y Cajal contract (RYC2019-026885-I) from the Spanish Ministry of Science and Innovation (MCIN).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: We would like to thank Vincenzo Biancone, Director of Monta Conca Reserve for allowing the research activities. We gratefully acknowledge the Centro Speleologico Etneo for the support and, in particular, the members F. Fiorenza, F. Leone, A. Marletta, A. Vasta. We thank the following specialists for the species identification: Amphipoda (F. Stoch), Annelida (Christer Erséus), Copepoda (Diana M. P. Galassi), Gastropoda (Fabio Liberto and Hans Boeters), Ostracoda (Ilaria Mazzini). We would also like to thank Viorel Atudorei (University of New Mexico) for conducting the isotopic analysis.

Conflicts of Interest: The authors declare no conflict of interest.

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Paper V

Article accepted in *Journal of Arachnology*

Meta menardi and *M. bourneti* (Araneae, Tetragnathidae) segregate along the altitudinal gradient of Mount Etna (Sicily, S–Italy)

Giuseppe Nicolosi[1], Elena Piano[1], and Marco Isaia[1]*

[1]Department of Life Sciences and Systems Biology, University of Turin, Via Verdi 8 - 10124, Torino, Italy
*Corresponding author: marco.isaia@unito.it

ABSTRACT

Meta menardi (Latreille, 1804) and *M. bourneti* Simon, 1922 (Araneae: Tetragnathidae) are ubiquitous inhabitants of the twilight zone of most hypogean sites across Europe. Recent observations in volcanic caves located in Sicily pointed out the presence of both species on the island, with *M. menardi* in a small number of caves. On the basis of the field investigations conducted in 2017–2021, we analyzed the distribution and the habitat frequency of the two species along the altitudinal gradient of Mount Etna. Our results demonstrate a significant effect of climate and altitude on the segregation of the two species, with *M. menardi* occurring exclusively in caves above 1200 meters with lower temperatures (mean ± SD = 10.1 ± 0.68°C) and higher precipitations (682 ± 19 mm). Conversely, *M. bourneti* prefers sites with higher temperatures (14.5 ± 2.1°C) and lower precipitations (606 ± 46 mm). No difference was highlighted with respect to substrate age, as both species occurred in both old and recent caves (from 350 to >1,000 years). In light of the ongoing global warming, a possible increase in temperature could favor the upshift of *M. menardi* towards upper areas which are subjected to high volcanic risk. In this regard, investigations on the response of *Meta* spiders to increases in temperature on the Etna volcano would be an asset to establish an appropriate management plan for the conservation of the newly discovered population of *M. menardi* in Sicily.

Keywords: Cave-dwelling spiders, conservation, niche segregation, subterranean habitats, lava caves

INTRODUCTION

Spiders are one of the most diverse and successful groups of terrestrial predators (Hormiga and Griswold, 2014). Competition among spiders with similar ecological requirements is minimized in different ways, including resource partitioning (Richardson et al. 2009) and temporal or spatial segregation (Nieto-Castañeda et al. 2009; Villanueva-Bonilla et al. 2019). Spatial separation along environmental gradients is particularly visible in highly dispersive species, whereas competition plays a far less important role for low dispersal ones, such as the most specialized subterranean organism due to their narrow thermal tolerance (Mammola et al. 2019). On the other hand, less specialized species as *Meta* spiders are able to disperse outside and colonize newly suitable areas over long distances (Mammola and Isaia 2017).

The genus *Meta* (Araneae: Tetragnathidae) in Europe is represented by two of the most frequent species that can be encountered in the twilight zone of caves and other hypogean sites: *Meta bourneti* Simon, 1922 and *Meta menardi* (Latreille, 1804). Both species show a widespread distribution, especially when compared to the most specialized subterranean spiders (Koponen 1993; Deltshev 2011; Mammola and Isaia 2017; Hesselberg 2021). Such broad ranges can be explained in light of their life cycle, which includes a sedentary hypogean phase and an epigean one (Mammola and Isaia 2014), allowing efficient dispersion across suitable habitats.

Meta bourneti is widely distributed in the Mediterranean basin (Nentwig et al. 2022), from Europe to Georgia and North Africa (World Spider Catalog 2022) and its ecological preference for warmer hypogean sites (above 9°C) makes it particularly abundant in Southern Europe. Conversely, *M. menardi* shows a higher occurrence for cooler hypogean sites (5–9°C) (Isaia et al. 2007; Mammola and Isaia 2014) and is mainly distributed in the Palaearctic region, reaching northward into the Scandinavian Peninsula and eastward Ukraine and Turkey (World Spider Catalog 2022).

In Italy both species are widely distributed (Pantini and Isaia 2019), with abundant populations of *M. bourneti* in the South and *M. menardi* in the North (Pantini and Isaia 2019).

In Sicily, the presence of *M. bourneti* has been widely documented since 1963 (Dresco 1963), whereas *M. menardi* has never been recorded so far.

In this paper we reviewed available literature data on *Meta* spiders in Sicily and present a number of original occurrences gathered in recent years, including the first record of *M. menardi* for the island, one of the southernmost populations in Europe. In light of the geographical isolation of the newly discovered population, the possible competition with *M. bourneti* driven by ongoing increasing global temperature, we also provide some insights about the ecological factors driving habitat segregation in these two species along the altitudinal gradient of Mount Etna.

MATERIALS AND METHODS

Literature survey.—We examined all available literature data referring to the presence of *Meta* spiders in Sicily. For the bibliographic survey, we referred to the reference list of the updated version of the Italian checklist of the Italian spiders (Pantini and Isaia 2019).

Field survey.—The field activities were set up on Mount Etna, an active stratovolcano of 3,350 m formed at the beginning of the Quaternary in northeastern Sicily (Rittman 1976), representing the highest peak on the island. More than 200 volcanic caves are censused on the Etna Volcano (Centro Speleologico Etneo 1999), opening at different altitudes and showing an extremely diversified range of ecological and microclimatic conditions. Caves closer to the sea coast are generally warm and dry, whereas caves opening nearby the summit of the volcano are cold and humid.

We investigated 27 caves opening at different altitudes and on different slopes of the volcano. Caves were selected among the most accessible based on both security of access and practicability of the galleries, aiming at minimizing risk in highly active sectors of the volcano due to frequent collapse of cave structures. Although they greatly vary in length, most caves developed horizontally. The presence of *Meta* spiders at each site was verified through visual census and occasional hand sampling along the main gallery of the lava tube.

Although *M. menardi* shows some seasonal migrations in the deeper cave sectors (Gasparo and Thaler 2000), and *M. bourneti* perform vertical movements along cave walls (Lunghi 2018), adult spiders were regarded as sedentary in the cave, with no evidence of migration outside the cave (Smithers 2005; Mammola and Isaia, 2014). Consequently, even a single sighting was considered as a valid occurrence point.

Field activities covered a period of five years, discontinuously from February 2017 to September 2021, covering all seasons, and at least twice per year. At each visit we spent at maximum of one hour searching for spiders.

Statistical analysis.—Environmental occurrences of *Meta bourneti* and *M. menardi* living in Mount Etna lava caves were tested using data on bioclimatic factors derived from WorldClim 2.1 generated on climate data across years 1970–2000 (https://www.world clim.org/; Fick and Hijmans 2017). Lava ages were obtained from Etna Volcano's Geological map (1:50,000 scale) (Branca et al. 2011).

We selected the following continuous covariates as potential variables explaining the presence of the two species in the study area: annual mean temperature (BIO1), annual precipitations (BIO12), elevation, and land use.

Data on the distribution and environmental frequency of the two species were elaborated in R environment (R Core Team 2021). Each variable was analyzed separately with the nonparametric Mann–Whitney test for non–normally distributed variables. Normality was tested using the ‘shapiro.test’ function from ‘stats’ package (R Core Team and Contributors Worldwide 2021).

RESULTS

The bibliographic survey led us to record the occurrence of *Meta bourneti* in 16 localities, encompassing 4 provinces (Appendix 1), while our field activities led to the discovery of a number of new records, mostly within the Regional Park of Mount Etna (EUAP0227) (Appendix 1, Fig. 1). For each cave, we report speleological cadastre numbers in square brackets when available/applicable [regional code, provincial code, cave number].

All faunistic data have been deposited in the Spider Trait Database (Pekár et al. 2021), accessible at https://spidertraits.sci.muni.cz/datasets/106.

Material.—***Meta bourneti* Simon, 1922:** CATANIA Prov. 1 ♀; Parco dell'Etna, Zafferana etnea, Grotta del Gatto; 37°40'59.0"N, 15°05'08.7"E; 06 Feb. 2017; Nicolosi leg.; 1 ♀; Parco dell'Etna, Trecastagni, Grotta di Monte Cicirello, [SiCT1156]; 37°40'11.2"N, 15°04'14.7"E; 06 Mar. 2017; Nicolosi leg.; 1 juv; Parco dell'Etna, Ragalna, Grotta Catanese I [SiCT1037]; 37°38'54.0"N, 14°56'21.0"E; 09 Jun. 2017; Nicolosi leg.; 1 ♀; Parco dell'Etna, Linguaglossa, Grotta di Piano Porcaria [SiCT1101]; 37°47'52.1"N, 15°06'28.8"E; 12 Nov. 2017; Nicolosi leg.; 1 ♀; Catania, Grotta dei Roditori; 37°31'55.7"N, 15°04'27.5"E; 16 Dec. 2017; Nicolosi vid.; 1 ♀; Catania, Grotta Lucenti; 37°30'47.4"N, 15°03'54.6"E; 01 Mar. 2018; Nicolosi vid.; 1 ♀; "Complesso Immacolatelle e Micio Conti" Nature Reserve [ITA070008], San Gregorio di CT, Grotta della Tesi; 37°33'40.7"N, 15°06'47.1"E; 11 Feb. 2019; Nicolosi vid.; 1 ♀; "Complesso Immacolatelle e Micio Conti" Nature Reserve, San Gregorio di CT, Grotta Micio Conti [SiCT016]; 37°33'39.5"N, 15°07'01.0"E; 11 Feb. 2019; Nicolosi vid.; 1 ♀; Parco dell'Etna, Ragalna, Grotta Catanese I [SiCT1037]; 37°38'54.0"N, 14°56'21.0"E; 21 Jan. 2021; Nicolosi vid.; 2 ♀; Parco dell'Etna, Trecastagni, Grotta di Monte Cicirello [SiCT1156]; 37°40'11.2"N, 15°04'14.7"E; 16 Jun. 2021; Nicolosi vid.; 1 ♀; Parco dell'Etna, Nicolosi, Grotta Lunga [SiCT1029]; 37°37'47.0"N, 15°02'08.4"E; 16 Jun. 2021, Nicolosi vid.; 2 ♀; Parco dell'Etna, Adrano, Grotta di San Nicola [SiCT1032]; 37°42'33.1"N, 14°52'33.2"E; 18 Jun. 2021, Nicolosi vid.; 1 ♀; Parco dell'Etna, Milo, Grotta Pietrabuca; 37°44'47.4"N, 15°05'21.1"E; 20 Jul. 2021; Nicolosi vid.; 3 ♀; Parco dell'Etna, Linguaglossa, Grotta di Piano Porcaria [SiCT1101]; 37°47'52.1"N, 15°06'28.4"E; 28 Jul. 2021; Nicolosi vid.; 1 ♀; Parco dell'Etna, Linguaglossa, Grotta del Porcospino [SiCT1033]; 37°47'54.0"N, 15°07'04.8"E; 28 Jul. 2021; Nicolosi vid.; 1 ♀; Mascali, Grotta Forcato [SiCT1013]; 37°46'49.3"N, 15°08'46.8"E; 04 Aug. 2021; Nicolosi vid.; 1 ♀; Parco dell'Etna, Pedara (Tarderia), Cava dell'Istrice; 37°38'58.1"N, 15°03'19.3"E; 07 Sep. 2021; Nicolosi vid.

SIRACUSA Prov. 1 ♀; "Complesso Speleologico Villasmundo–S. Alfio" Regional Nature Reserve [EUAP1147], Melilli, Grotta Villasmundo [SiSR7032]; 37°13'00.9"N, 15°06'01.4"E; 02 Dec. 2017; Nicolosi leg.

TRAPANI Prov. 1 ♀; Santa Ninfa, Grotta di Santa Ninfa, "Grotta di Santa Ninfa" Nature Reserve; 37°46'50.1"N, 12°54'46.9"E; 28 Feb. 2017; Isaia and Nicolosi leg.

***Meta menardi* (Latreille, 1804)**: CATANIA Prov. 1 ♀; Parco dell'Etna, Bronte, Grotta dei Ragazzi; 37°45'57.0"N, 14°55'32.7"E; 09 Sep. 2021; Nicolosi vid.; 1 ♀; Parco dell'Etna, Randazzo, Grotta del Nano; 37°49'23.6"N, 14°58'44.9"E; 29 Aug. 2021; Nicolosi vid.; 1 ♀; Parco dell'Etna, Randazzo, Grotta del Faggio; 37°49'19.5"N, 14°58'43.4"E; 29 Aug. 2021; Nicolosi vid.; 1 ♀; Parco dell'Etna, Castiglione di Sicilia, Grotta delle Femmine [SiCT1046]; 37°49'31.6"N, 15°01'24.9"E; 03 Aug. 2021; Nicolosi vid.; 1 ♀; Parco dell'Etna, Ragalna, Grotta dell'Immacolata [SiCT244]; 37°40'49.1"N, 14°57'31.7"E; 08 Jul. 2021; Nicolosi vid.; 3 ♀; Parco dell’Etna, Belpasso, Grotta di Monte Giacca [SiCT1145]; 21 Jun. 2021; 37°40'58.3"N, 14°59'12.4"E; Nicolosi vid.; 1 ♀; Parco dell’Etna, Adrano, Grotta di Monte Gallo [SiCT1176]; 37°43'32.0"N, 14°54'50.3"E; 18 Jun. 2021; Nicolosi vid.; 1 ♂; Parco dell’Etna, Adrano, Grotta di Monte Intraleo [SiCT1007]; 37°43'13.0"N, 14°54'33.0"E; 18 Jun. 2021; Nicolosi vid.; 2 ♀; Parco dell’Etna, Linguaglossa, Grotta di Monte Crisimo [SiCT1170]; 37°47'42.5"N, 15°05'15.2"E; 13 Jun. 2021; Nicolosi vid.; 2 ♀; Parco dell’Etna, Castiglione di Sicilia, Grotta di Monte Corruccio [SICI1056]; 37°48'37.4"N, 15°04'30.6"E; 08 Jun. 2021; Nicolosi vid.; 1 ♀; Parco dell’Etna, Sant’Alfio, Grotta della Neve [SiCT1117]; 37°46'16.9"N, 15°04'17.5"E; 08 Jun. 2021; Nicolosi vid.; 1 ♀; Parco dell’Etna, Randazzo, Grotta Piano Cavoli; 37°50'29.0"N, 14°59'29.0"E; 02 Jun. 2021, Nicolosi vid.; 1 ♀; Parco dell’Etna, Maletto, Grotta del Cernaro [SiCT1068]; 37°48'13.0"N, 14°55'44.0"E; 29 Nov. 2017; Nicolosi leg.; 1 ♂; Parco dell’Etna, Adrano, Grotta di Monte Intraleo [SiCT1007]; 37°43'13.0"N, 14°54'33.0"E; 18 Nov. 2017; Nicolosi leg.; 1 ♀; Parco dell’Etna, Piedimonte etneo, Grotta di Piano Noce; 37°47'18.1"N, 15°06'18.3"E; 12 Nov. 2017; Nicolosi leg.; 1 juv.; Parco dell’Etna, Sant'Alfio, Grotta dei Ladri [SiCT1204]; 37°46'17.7"N, 15°04'18.7"E; 29 Oct. 2017; Isaia and Nicolosi leg.

Habitat occurrence.—Our field activities led us to record the presence of *Meta bourneti* in 19 caves, whereas the presence of *M. menardi* was recorded in 14 caves.

As expected, according to the general preference for the twilight zone of *Meta* spiders (Mammola and Isaia 2014; Smithers 2005; Lunghi 2018; Mammola and Isaia 2017) most individuals occurred in the vicinity of the cave entrance.

Our results support a significant effect of altitude (Mann–Whitney test: N = 33, W = 266, $p < 0.001$), temperature (N = 33, W = 266, $p < 0.001$) and precipitation (N = 33, W = 251, $p < 0.001$) in determining the segregation of the two species along the considered altitudinal gradient.

Accordingly, *Meta menardi* colonizes caves above 1,200 meters a.s.l. (mean ± SD = 1441 ± 130 m) while *M. bourneti* occurs more frequently at lower altitudes (656 ± 372 m) (Fig. 2A-D). Neither of the species occurred over 1,600 meters and were never found to occur in the same caves. The minimum distance between a site colonized by *M. bourneti* and one by *M. menardi* is approximately at a linear distance of 1 km and an altitude difference between sites of 100 m.

In particular, when considering our results in terms of cave temperatures, *M. menardi* occurs more frequently in colder sites (mean ± SD = 10.1 ± 0.68°C) and with higher precipitation (682 ± 19 mm) compared to *M. bourneti,* occurring in sites with higher temperature (14.5 ± 2.1°C) and with lower precipitation (606 ± 46 mm).

Lastly, both species seem able to colonize lava caves irrespectively to lava age, occurring both in sites of recent and old formation (from 350 to >1,000 years). Accordingly, the Mann–Whitney test did not show any significance in respect to this factor (N = 33, W = 85, $p > 0.05$).

DISCUSSION

Recent research on subterranean organisms proved their sensitivity to microclimatic fluctuations (Culver and Pipan 2019; Nicolosi et al. 2021). Accordingly, sensitivity is particularly high in spiders characterized by high development of troglomorphic traits, which exhibit low ranges of thermal tolerance (Mammola et al. 2019, 2020).

Although *Meta bourneti* and *M. menardi* are not strictly subterranean, a certain relation with microclimate has been proved to be one of the most important factors determining niche segregation in these spiders (Mammola and Isaia 2014 but see also Brignoli 1971; Ribera 1978; Gasparo and Thaler 2000 for further evidence).

Based on literature sources and recent field activities, in this work we reported new occurrences of *Meta* spiders in Sicily, with particular emphasis on the first regional record of *M. menardi* and on the ecological requirements of the two species along the altitudinal gradient of Mount Etna.

M. bourneti was previously known in Sicily for several locations. The species was first detected by Dresco 1963 in “Grotte di Baida”, in the province of Palermo. Subsequently, records of the species were reported from the province of Trapani, Siracusa, and Catania (Brignoli 1979), including the occurrence of the species in a few lava caves of Mount Etna.

Our field investigations on Mount Etna led to the discovery of several new occurrences of *M. bourneti* and a new record of *M. menardi* for Sicily. Our new record represents the southernmost population of the species in Italy and figures among the southernmost in Europe (Nentwig et al. 2022).

Meta menardi is here reported from 14 caves distributed along the slopes of Mount Etna (Fig. 1). The species colonizes caves above 1,200 m a.s.l. reaching the maximum altitude of 1,600 m, confirming its higher frequency for colder and wettest sites seen in Mammola and Isaia 2014. Instead, *M. bourneti* generally occurs at lower altitudes, with records spanning from the coastline up to 1,200 m, confirming its ability to tolerate a wider range of climatic conditions.

A possible mechanism favoring the coexistence of spiders in subterranean environments is represented by the shift in the trophic and temporal niche (Novak et al. 2010). Pavlek and Mammola (2021) suggested that food specialization in subterranean spiders may be represent a possible way to avoid direct competition. Moreover, Mammola et al. (2016) pointed out dynamic partitioning of the spatial niche of troglophile spiders occurring in synotopy in the twilight zone of Western Alpine caves. As for *Meta* spiders, Mammola and Isaia (2014) demonstrated a complete niche partitioning between *M. menardi* and *Meta bourneti*, achieved through conditional differentiation, with *M. menardi* more competitive at narrow ranges of cooler temperatures and higher relative humidity.

Accordingly, we have never observed the two species inhabiting the same cave, despite the small distribution of *M. menardi* on Mount Etna and the short distance between sites of occurrences.

As far as our data show, the two species get the closest within the SIC “Monte Baracca e Contrada Giarrita” (ITA070014) in Grotta di Piano Porcaria (with *M. bourneti* at 1099 m a.s.l.) and Grotta di Piano Noce (with *M. menardi*, 1195 m), located approximately at a linear distance of 1 km one from the other. It goes without saying that this location represents an ideal system where to monitor the possible replacement of *M. menardi* by *M. bourneti,* especially in the perspective of the ongoing temperature rise due to climate change.

Neither of the species seems able to colonize volcanic caves of Mount Etna over 1,600 meters, probably due to unfavorable microclimatic conditions or, more likely, to the lack of suitable habitat in the high volcanic areas for the survival of the epigean juvenile stages. The latter seems a reasonable hypothesis given that this species has been observed (Isaia et al. 2011, Mammola et al. 2021) in caves opening in vegetated areas (pastures) up to 2,000 m. Moreover, Smithers 2005 has noticed a seasonal increase in the abundance of juvaniles climbing on the vegetation growing in the vicinity of a mine entrance, attesting the role of vegetation in determining higher habitat suitability for *Meta* menardi. Tentatively, caves located at higher elevations on Mount Etna may represent a hostile environment for the epigean juvenile phase of *M. menardi*, due to the presence of lava substrates devoid of vegetation and frequently affected by volcanic activities (i.e., volcanic ash fall, presence of lava flows). These conditions may hamper dispersal and inhibit the expected elevational upshift of the species due to the ongoing temperature increase, posing a serious threat to the survival of the Sicilian populations of *M. menardi*. In turn, species replacement may occur at lower altitudes, where sites abandoned by *M. menardi* may become suitable for *M. bourneti*.

CONCLUSIONS

Due to the scarce biospeleological research activities in Sicily (but see, Caruso 1982; Sendra et al. 2019; Sabella et al. 2019, 2020), the new record of *Meta menardi* on Mount Etna represents an important outcome from both the faunistic and the ecological point of view, especially considering the lack of arachnological investigations on the island and the high level of isolation of this population. Considering the higher frequency of *M. menardi* for cold caves (around 10°C), we hypothesize that the ongoing climatic change may significantly alter subterranean climatic conditions leading to a possible reduction of the Sicilian population of *M. menardi* and a corresponding expansion of the local range of *M. bourneti.*

Further investigations on the response of *Meta* spiders to increases in temperature on the Etna volcano (Sicily) would be therefore an asset to establish an appropriate management plan for the conservation of the newly discovered population of *M. menardi* in Sicily.

ACKNOWLEDGMENTS

We thank the “Ente Parco dell’Etna” that authorized and allowed the carrying out of the scientific research activity in the Park.

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APPENDIX 1

Occurrences of *Meta bourneti* Simon, 1922 and *M. menardi* Latreille, 1804 in Sicily, with details on localities and source of reference.

Locality [cadastral number]	**Municipality (Province)**	**Reference**
Meta bourneti		
Grotta di Santa Ninfa [SiTP8000]	Santa Ninfa (TP)	Brignoli 1979; Caruso 1982
Grotta Scannato [1060SiCT]	Scillichenti, Acireale (CT)	Brignoli 1979; Caruso 1982; Caruso 1999
Grotta Immacolatella I [SiCT1015]	San Gregorio (CT)	Brignoli 1979; Caruso 1982; Caruso 1999
Grotta di Maniace [SiCT1098]	Maletto (CT)	Brignoli 1979; Caruso 1982; Caruso 1999
Grotta delle Balze Soprane I [SiCT131]	Bronte (CT)	Brignoli 1979; Caruso 1982; Caruso 1999
Grotta del Fico I [SiCT1081]	San Gregorio (CT)	Brignoli 1979; Caruso 1982; Caruso 1999
Grotta Scrivilleri [SiSR7003]	Priolo Gargallo (SR)	Brignoli 1979; Caruso 1982
Grotta San Marco [SiSR3512]	Noto (SR)	Brignoli 1979; Caruso 1982
Sotto il convento di Baida	Palermo (PA)	Dresco 1963; Caruso 1982
Inghiottitoio di Monte Conca [SiCL3000]	Campofranco (CL)	Nicolosi et al. 2022
Grotta Villasmundo [SiSR7032]	Melilli (SR)	Original data
Grotta Forcato	Mascali (CT)	Original data
Grotta Catanese I [SiCT1037]	Ragalna (CT)	Original data
Grotta del Gatto	Zafferana (CT)	Original data
Grotta del Porcospino [SiCT1033]	Linguaglossa (CT)	Original data
Grotta di Monte Cicirello	Trecastagni (CT)	Original data
Grotta di Piano Porcaria [SiCT1101]	Linguaglossa (CT)	Original data
Grotta di San Nicola [SiCT1032]	Adrano (CT)	Original data
Grotta Lunga [SiCT1029]	Nicolosi (CT)	Original data
Grotta Pietrabuca	Milo	Original data
Grotta dei Roditori	Catania (CT)	Original data
Grotta Micio Conti [SiCT016]	San Gregorio (CT)	Original data
Grotta della Tesi	San Gregorio (CT)	Original data
Grotta Lucenti	Catania (CT)	Original data
Cava dell'Istrice	Tarderia, Pedara (CT)	Original data
Meta menardi		

Grotta di Piano Noce	Linguaglossa (CT)	Original data
Grotta di Monte Crisimo [SiCT1170]	Linguaglossa (CT)	Original data
Grotta di Monte Intraleo [SiCT1007]	Adrano (CT)	Original data
Grotta di Monte Corruccio [SICI1056]	Castiglione di Sicilia (CT)	Original data
Grotta del Cernaro [SiCT1068]	Maletto (CT)	Original data
Grotta dell'Immacolata [SiCT244]	Ragalna (CT)	Original data
Grotta di Monte Gallo [SiCT1176]	Adrano (CT)	Original data
Grotta di Monte Giacca [SiCT1145]	Belpasso (CT)	Original data
Grotta della Neve [SiCT1117]	Sant’Alfio (CT)	Original data
Grotta delle Femmine [SiCT1046]	Castiglione di Sicilia (CT)	Original data
Grotta Piano Cavoli	Randazzo (CT)	Original data
Grotta del Nano	Randazzo (CT)	Original data
Grotta del Faggio	Randazzo (CT)	Original data
Grotta dei Ragazzi	Bronte (CT)	Original data

Paper VI

Article in preparation

Future shifts of cave-dwelling *Meta* spiders under the influence of global warming on Etna volcano, Sicily

Giuseppe Nicolosi[1], Elena Piano[1], Emanuela De Beni[2] & Marco Isaia[1]

[1]Department of Life Sciences and System Biology, University of Torino, Torino, Italy

[2]Istituto Nazionale di Geofisica e Vulcanologia, Catania, Italy

ABSTRACT

Meta menardi (Latreille, 1804) and *M. bourneti* Simon, 1922 (Araneae: Tetragnathidae) are ubiquitous inhabitants of the twilight zone of most hypogean sites across Europe. Despite both species being mostly distributed in subterranean habitats, they show broad ranges of distribution compared to most specialised subterranean spiders which can be explained in the light of their life cycle that includes an epigean and hypogean ecophase.

Data on the occurrences of both *Meta* spiders on Mount Etna lava caves were recently gathered by means of sampling activities. Sixty-nine caves were surveyed on Mount Etna opened at different altitudes and on different slopes of the volcano.

We used species distribution modelling (SDM) to evaluate potential effects of climate change on both *M. bourneti* and *M. menardi* on Mount Etna. Moreover, we evaluated the width of the bioclimatic niche of the two species and assessed the potential overlap between their multidimensional Hutchinsonian hypervolumes.

Three common models in SDM for presence-absence data were used for modelling species distributions to predict current and future (2070) suitable areas of both species.

The overall size of the niche hypervolumes of the two species has shown a markable smaller bioclimatic niche of *M. menardi* compared to *M. bourneti*. Moreover, the niche hypervolume of M. menardi results almost completely comprised that of *M. bourneti*, suggesting the ability of both species to exploit a similar variety of microhabitats.

On the base of SDM analysis, we predicted a suitable area predicted by the models for both species in accordance with its current range distribution on Mount Etna. According to SDM result, *M. bourneti* is distributed from the sea level up to 1100 meters, whereas *M. menardi* along a narrow strip on Mount Etna, between the areas colonised by *M. bourneti*, and those sites at higher altitudes interested by recent volcanic activity.

The averaged future predictions from the three climatic models for 2070 under RCP 8.5 scenario, showed that both species will be affected by climate change with *M. bourneti* expanding its

distribution toward new suitable areas up to 1,300 meter favored by the increase of temperature. The predictions for *M. menardi* would instead potentially lead to the abandonment of southern caves on Mount Etna, causing a significant loss of suitable areas, likely determining the disappearance of the species from the study area due to the global change. Future intervention would be need to plan for the conservation of the population of *M. menardi* in Sicily.

INTRODUCTION

Subterranean ecosystems are extremely stable environments to their surrounding external environments (Poulson & White, 1969; Howarth, 1993). The species living in these habitats often show pronounced sensitivity to even small changes in their environmental conditions (e.g., Barr & Kuehne, 1971; Howarth, 1980; Mammola et al., 2019; Paper II). Thus, subterranean species may not be able to adapt to climate change, but rather can theoretically cope with climatic alterations only by shifting their distributional range (Mammola & Isaia, 2017).

This issue is particularly evident in the case of low dispersal animals like highly specialised species to subterranean habitats, given their reduced physiological tolerance (e.g., Novak et al., 2014), which represents a limit in their dispersal ability (e.g., Cardoso, 2012; Mammola, Isaia & Arnedo, 2015).

Cave-dwelling invertebrates with poor subterranean adaptations have instead retained the ability to disperse effectively outside the caves in certain phases of their development. This is well-exemplified by the troglophile cave-dwelling spiders *Meta menardi* (Latreille) and *M. bourneti* Simon (Araneae: Tetragnathidae), ubiquitous inhabitants of the twilight zone of most hypogean sites across Europe.

Despite both species are mostly distributed in subterranean habitats, they show broad ranges of distribution compared to most specialised subterranean spiders (Deltshev, 2011; Koponen, 1993; Mammola & Isaia, 2017), which can be explained in the light of their life cycle (Mammola & Isaia, 2014). The life cycle of both species includes an epigean ecophase in which the spiderlings, after the first molt, move towards the cave entrance and disperse via ballooning outside the cave (Tercafs, 1972; Smithers & Smith, 1998; Smithers, 2005).

The ecological requirements of *M. menardi* and *M. bourneti* are very similar, when the microclimatic conditions in a cave are favorable for *M. menardi* - narrow ranges of low temperature and high humidity - *M. bourneti* is excluded by competition (Mammola & Isaia, 2014). On the contrary, a possible increase in temperature can instead favour the expansion of *M. bourneti* (Mammola & Isaia, 2017). Accordingly, *M. bourneti* shows a preference for warmer hypogean sites (above 9°C) and is particularly abundant in Southern Europe being widely distributed in the Mediterranean basin (Nentwig et al. 2022), from Europe to Georgia and North Africa (World Spider Catalog 2022). Conversely, *M. menardi* shows a higher occurrence for cooler hypogean sites (5–9°C) (Isaia et al. 2007; Mammola and Isaia 2014) and is mainly distributed in the Palaearctic region, reaching northward into the Scandinavian Peninsula and eastward Ukraine and Turkey (World Spider Catalog 2022).

Although Sicily has a speleological variety of caves excavated or formed within limestone, volcanic or gypsumrocks and large karstic areas, its subterranean fauna is still poorly known (Cobolli et al. 1994). Recent observations on the Etna lava tubes (Paper V) pointed out for the first time on the island the presence of *M. menardi* distributed along the slopes of Mount Etna in caves above 1,200 m a.s.l. reaching the maximum altitude of 1,600 m, confirming its higher frequency for colder and wettest sites seen in Mammola and Isaia 2014. On the contrary, *M. bourneti* prefers warmer sites, with records spanning from the coastline up to 1,200 m. Neither of the species have been observed over 1600 meters. Considering the occurrence of both species in caves located up to 2000 meters in other localities, this trend for *Meta* spiders on Mount Etna is likely ascribed to the unsuitable habitat in the high volcanic areas of Mount Etna (i.e. presence of lava substrates devoid of vegetation and frequently affected by volcanic activities), especially for the survival of the epigean juvenile stages (Paper V).

In light of the high dispersal ability of both species, *Meta* spiders would easily come into contact in a global warming scenario, especially in light of the small range of distribution of *M. menardi* on Mount Etna and that the species have never been observed to coexist within the same hypogean site (Brignoli, 1971, 1979; Gasparo & Thaler, 2000; Milner, 2013; Ribera, 1978).

Thus, in the light of a possible rise in temperature, species replacement may occur at lower altitudes, where the limited population of *M. menardi* is destined to be replaced by the *M. bourneti* species, causing its local extinction in Sicily. A better understanding of the distribution and environmental requirements of the genus *Meta* living on Mount Etna is therefore required to estimate the current and future range of both species in light of global warming scenarios, also to establish more appropriate management plans for their conservation, both at local and global scale. It represents a locality of interest as islands (Veron et al., 2019) and mountains (Thuiller, 2007) are generally more vulnerable to climate change, as conditions that may hamper the dispersion towards suitable areas.

The well-defined microclimatic range makes the genus *Meta* an interesting target of research and a suitable model for the study of the effect of climatic changes. The selected species are hence good indicators at a local scale, suitable to determine the response to climate change.

In this regard, we carried out investigations on Etna lava caves aiming at i) characterising the main factors of both Meta spiders in order to identify the main driver explaining the presence and absence on the Etna volcano lava caves; ii) investigate the environmental factors explaining the niche segregation of the two species; iii) predict the future rearrangement in the species distribution under the future warming scenarios at regional scale.

MATERIALS AND METHODS

Study area

The field activities were set up on Mount Etna, a volcano that spans 3350 m in elevation and formed at the beginning of the Quaternary in the northeastern part of Sicily (Rittman, 1976), representing the highest peak in Sicily.

The volcano has a total area of approximately 2,100 km^2 delineated by the Regional Park of Mount Etna (EUAP0227). It is located 20 km north of the city of Catania (15°0′E, 37°43.8′N) and represents one of the most active volcanoes in the world (Branca & Del Carlo, 2004). The high frequency of volcanic activities, both effusive and explosive, and the opening of eruptive fissures on the flanks of the volcano, as the 1991-1993 eruption (e.g., Calvari et al., 1994), causes continuous modifications of the slopes, especially in the summit areas (Bisson et al., 2021). Due to its geographical, geological and climatic isolation, Mount Etna hosts a high diversity of endemic plants (Sciandrello et al., 2020). Vegetation on Mount Etna is frequently affected by volcanic activities causing changes in structure, density, cover, floristic composition, species richness and diversity (Poli & Grillo, 2000). Gradually, with time, the youngest lava fields are slowly colonised. Lichens or bryophytes are beginning to appear and once the colonisation proceeds, the vegetation cover gradually increases and the shrubs begin to become organised into communities. At lower elevations, the communities are most frequently dominated by *Spartium junceum*, at higher elevations by *Genista aetnensis* (Poli & Grillo, 2000).

Over 200 lava tubes are known around the volcano (Centro Speleologico Etneo 1999), opening at different altitudes and showing an extremely diversified range of ecological and microclimatic conditions. Caves closer to the sea coast are generally warm and dry, whereas caves opening nearby the summit of the volcano are cold and humid. Although lava tubes are normally associated with pahoehoe lavas, on Etna they formed in both `a`a and pahoehoe flows as well documented (e.g. Calvari, 1999).

Species distributional data

The distribution of *Meta bourneti* in several lava caves of Mount Etna is well documented (e.g., Caruso & Costa, 1978; Caruso, 1982, 1995), whereas the presence of *M. menardi* has been recently spotted in a limited number of caves (Paper V).

Data on the absence of both species were obtained through sampling activities performed on Mount Etna during the period 2017-2021. The final dataset of occurrences was verified from the updated version of the Italian checklist of the Italian spiders (https://www.araneae.it/, accessed on 15 December 2022), and the Spider Trait Database (https://spidertraits.sci.muni.cz/datasets/106, accessed on 15 December 2022).

Data extraction

We extracted the land use at each sampling site on digital maps in QGis (Quantum Gis Development 2022) in a buffer of 50 m radius with the sampling site at the centre. Land use was obtained from regional data (available at www.sitr.regione.sicilia.it) and we calculated the percentage of the following three land use categories: **vegetated areas, lava fields and anthropogenic use**. The category “vegetated areas” incorporated all the land uses referring to vegetation, from woodlands to prairies, while “anthropogenic use” included both agricultural and urban areas. Lava fields refers to those areas that are covered with recent lava flows still not covered by vegetation.

Bioclimatic data were obtained from the WorldClim website (www.worldclim.org). We downloaded the average data for the years 1970-2000 of 19 bioclimatic variables with a spatial resolution of 30 seconds. For each sampling site, we extracted the value of each bioclimatic variable by using the function “extract” from the package *raster* (Hijmans, 2015) in the R software (R Core Team 2021).

Niche hypevolume

All statistical analyses were performed with R software (R Core Team, 2021).

In a first step, we reduced collinearity among bioclimatic variables and elevation by performing a Principal Component Analysis (PCA) using the ‘princomp’ function. Based on the scree plot (Fig. S1), we retained the first three axes (hereafter PC1, PC2 and PC3) that explained around 96.5% of the total variance (PC1 = 81%; PC2 = 9.6%; PC3 = 5.8). Then, we tested for multicollinearity among the PCA axes and land use variables with the R Pearson test (Fig. S2). As all variables were highly correlated (|R| > 0.5), only the three PCA axes were retained for the analysis of the hypevolume.

In a second step, a Permutational Multivariate Analysis of Variance based on Euclidean distances (PERMANOVA, Anderson, 2001) was then applied to the matrix obtained with the first four PCA axes to test for differences among the presence sites of the two *Meta* species and the absence sites with the function “adonis” from the *vegan* package (Oksanen et al. 2019). Statistical significance was tested via 9999 random permutations.

The bioclimatic niches of the two *Meta* species based on presence data were then investigated to highlight potential differences in their bioclimatic requirements. To perform this, we calculated their ecological niche *sensu* Hutchinson (1957) via the *hypervolume* R package (Blonder, 2015) based on the first four axes obtained with PCA. This approach quantifies the multidimensional Hutchinsonian hypervolume of a species based on a kernel density estimation (KDE) procedure performed on the standardised values of the environmental variables recorded in sites where the species is present (mathematical details in Blonder et al., 2014). The hypervolumes of the two *Meta* species were calculated with the hypervolume_gaussian R command (Blonder, 2015), which constructs a hypervolume based on a Gaussian KDE. Following the approach proposed by Blonder et al. (2014), we standardised the choice of bandwidth for each variable through a Silverman estimator (Silverman, 1992) and we set a threshold that included 100% of the total probability density. The overlap between the hypervolumes of the two species were obtained via the hypervolume_set and hypervolume_overlap_statistics R commands respectively (Blonder, 2015). Overlap statistics include the Jaccard and Sorensen similarity indices, which range from 0 to 1 (0 = no overlap; 1 = complete overlap).

Species Distribution Models (SDM)

Environmental predictors

We modelled the current distribution of the species *Meta bourneti* and *M. menardi* spiders in the study area by employing 19 bioclimatic variables and elevation data at a resolution of 2.50 (nearly 4.5 km at the equator) to represent current climatic conditions (1950–2000; Hijmans, Cameron, Parra, Jones & Jarvis, 2005; resolution: 30’’; available at: www.worldclim.org; Table S1). Data layers at the same spatial resolution were obtained for future scenarios (2070). The data available here are climate projections from the general circulation model (GCM) CCSM4 that were downscaled and calibrated (bias corrected) using WorldClim 1.4 as baseline climate. For CMIP5 we used the representative concentration pathway (RCP) with a high-emission scenario (RCP 8.5) scenario, which corresponds to the pathway with the highest greenhouse gas emissions (Wayne, 2013).

The collinearity among bioclimatic layers was reduced by performing a variance inflation factor (VIF) (Marquardt 1970) analysis by using the vif() function of the ‘usdm’ package (Naimi, 2015). A predictor was excluded for VIF values higher than 10 as it generally indicates a strong collinearity among variables (Chatterjee & Hadi 2006). After checking for collinearity, we reduce the full set of 19 environmental variables to 5 (BIO07 Temperature Annual Range, BIO08 Mean Temperature of Wettest Quarter, BIO16 Precipitation of Wettest Quarter, BIO18 Precipitation of Warmest Quarter, BIO19 Precipitation of Coldest Quarter).

Modelling procedures

Modelling was carried out in the R 4.2.1 statistical programming environment (R Core Team 2021). We performed for both species 3 commonly used models in SDM for presence-absence data implemented in the ‘sdm’ package (Naimi & Araújo, 2016). Models included a regression method: generalised linear models (GLM) (McCullagh and Nelder, 1989); a machine learning method: boosted regression tree (BRT) (Elith et al. (2008); a classification method: flexible discriminant analysis (FDA) (Hastie et al., 1994). For modelling, the occurrence points of one species were considered as true absence of the other species, and vice versa.

Models’ validations were performed by using the subsampling replication approach with the ‘sdm’ package developed by Naimi and Araújo (2016). In order to get independent validation statistics, 80% of the collected data was used to train models while 20% was used to validate them. We used the area under the Receiving Operator Curve (AUC) to evaluate model performance. The value of AUC ranges from 0 to 1 (Fielding and Bell, 1997). The mean AUC across the fifty replicates of each algorithm across all species was used to assess model performance. An AUC value of 0.50 indicates that model did not perform better than random, whereas a value of 1.0 indicates perfect discrimination (Swets, 1988).

Each SDM fitted using the current climate data was projected into the future climate scenarios. The mean habitat suitability was calculated for each current and 2070 climate-model scenario. We subsequently compared the habitat suitability for both current and 2070 future predicted climate scenarios under the CMIP5 (RCP 8.5) to show the increase (or decrease) of habitat suitability of both species.

The ‘sdm’ package was used to combine the distribution maps using the “ensemble” function to produce consensus ‘ensemble’ maps based on weighted AUC values (Naimi and Araújo, 2016). Ensemble prediction is a process of synthesising integrating multiple individual models provide robust estimates of potential species’ distributions (e.g., Araujo and New, 2007; Marmion et al., 2009; Meller et al., 2014; Poulos et al., 2012; but see Crimmins et al. 2013; Zhu and Peterson 2017).

RESULTS

The database included 34 presence sites of *Meta* species (19 presence sites for *M. bourneti* and 15 presence sites for *M. menardi*) and 35 absence sites (Fig. 1).

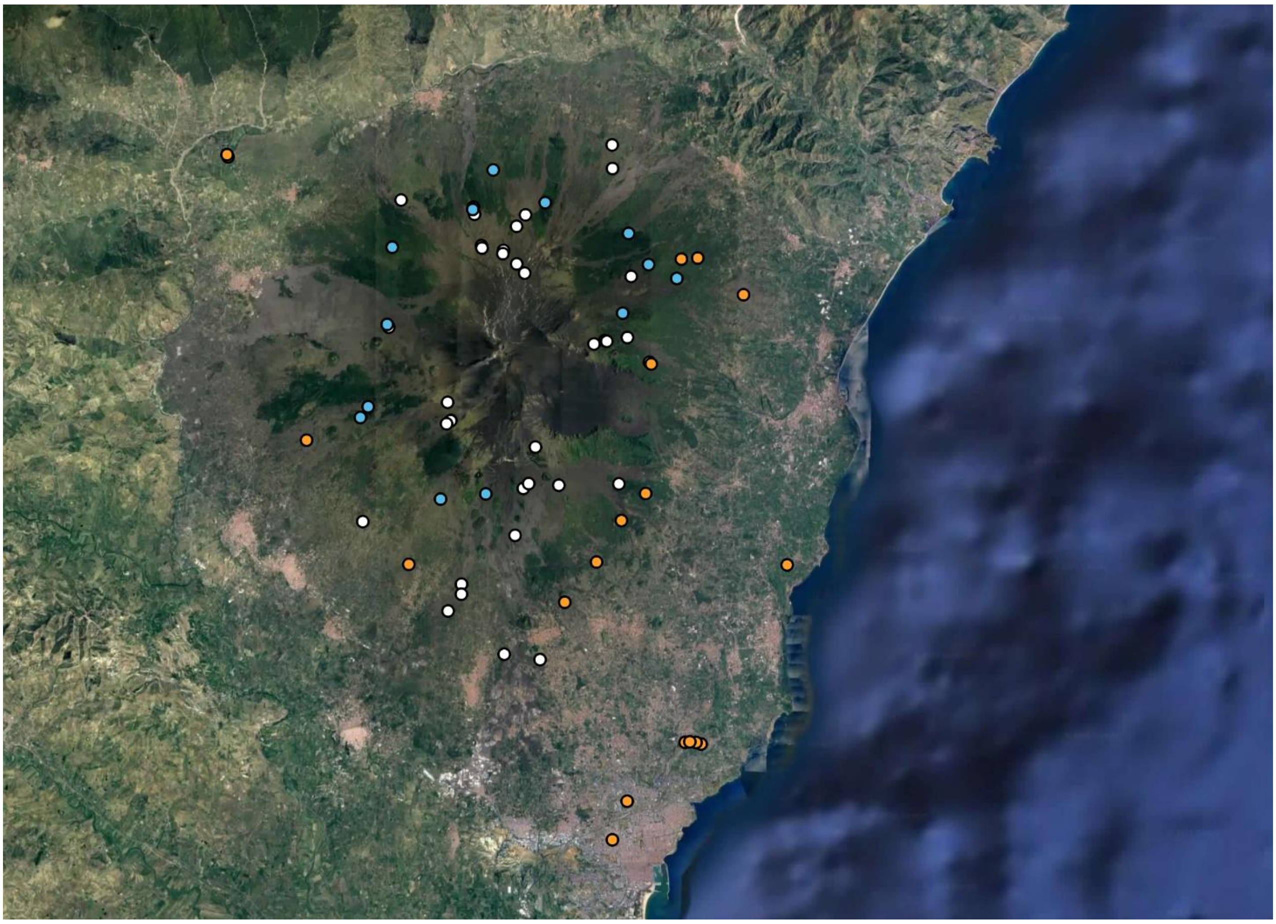

Figure 1. Map showing all occurrence records for *Meta bourneti* (orange) and *M. menardi* (blue) on Mount Etna (S-E Sicily). White spot indicates those sites of true absence of both species.

PCA

By visually inspecting the results of the PCA (Fig. 2), we could observe a clear separation between the presence sites of *M. menardi* and *M. bourneti,* as well as with absence sites, that was confirmed by the results of the PERMANOVA ($F_{2,66}$ = 21.7; P < 0.001).

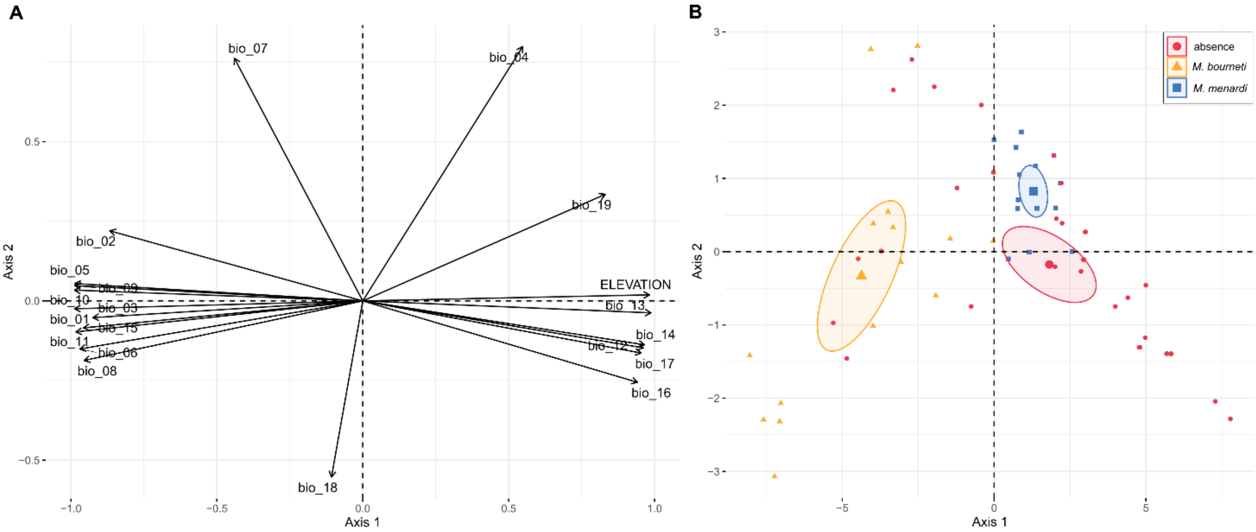

Figure 2. results of the PCA performed on the bioclimatic variables and elevation with the arrows representing the loadings of the variables to the first two axes (A) and points representing the scores of each sampling site (B). Ellipses represent standard deviations around the centroids of the three groups of sampling sites (red = absence sites; orange = presence of *M. bourneti*; light blue = presence of *M. menardi*).

By analysing the niche hypervolumes, *M. bourneti* showed the highest dimension of the four-dimensional hypervolume (1461.0), whereas the hypervolume of *M. menardi* was around 45-fold smaller (31.4). The visual inspection of the overlap between the two hypervolumes (Fig. 3) showed that the bioclimatic niche of *M. menardi* is almost completely comprised into that of *M. bourneti* as demonstrated by the unique fractions of the two hypervolumes (Jaccard = 0.02, Sorensen = 0.04; unique fraction *M. bourneti* = 0.98; unique fraction *M. menardi* = 0.02).

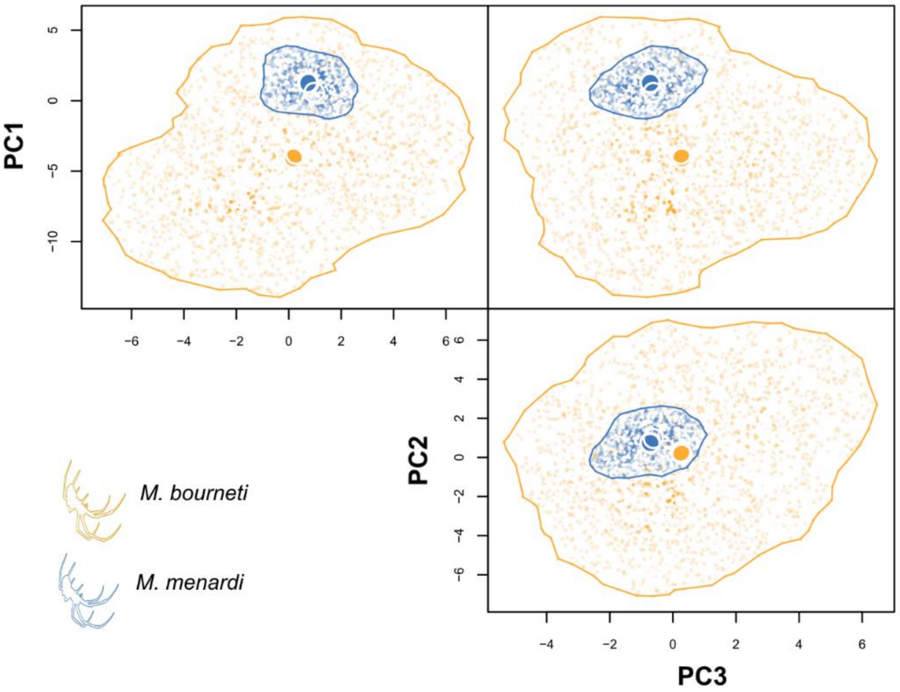

Figure 3. Pair plots showing the estimated three-dimensional hypervolumes for the two *Meta* species (orange = *M. bourneti*; light blue = *M. menardi*). The colored points for each taxon reflect the centroids (large points), original observations (intermediate points) and the stochastic points sampled from the inferred hypervolume (small points).

Model performance

The performances of SDMs using different evaluation techniques are presented in Table 1. Most of the individual models performed well in all evaluation metrics employed. Of the three modelling algorithms the predictive accuracies of AUC were higher than 0.8 in *Meta bourneti*. The GLM model showed the highest AUC with 0.91, followed by BRT (0.9) and FDA (0.87).

For *Meta menardi*, the predictive accuracies of AUC were higher than 0.8. The BRT showed the highest AUC with 0.88, followed by FDA (0.82) and GLM (0.81) (Table 1).

Species	**Method**	**AUC**	**COR**	**TSS**	**Deviance**
M. menardi	GLM	0.81	0.5	0.7	0.97
M. menardi	BRT	0.88	0.65	0.8	0.89
M. menardi	FDA	0.82	0.49	0.8	0.93
M. bourneti	GLM	0.91	0.64	0.83	0.78
M. bourneti	BRT	0.9	0.68	0.82	0.79
M. bourneti	FDA	0.87	0.6	0.75	0.92

Table 1. Evaluation metrics for Meta menardi and M. bourneti, including AUC (Area Under the Curve), PCC (Percent Correctly Classified), Sensitivity, Specificity, and TSS (True Skill Statistic) for each model algorithm (Boosted Regression Tree [BRT], Generalized Linear Model [GLM], and Flexible Discriminant Analysis [FDA]).

Environmental variables and response curves

The relative influence of predictors across models is shown in Figure S3. For *Meta bourneti*, the temperature of wettest quarter (BIO08) contributed most to all models with respectively 78.7% (GLM), 53.8% (BRT) and 59.1% (BRT). For *Meta menardi*, the Temperature Annual Range (BIO07) contributed most to the model GLM (33.5%), whereas the Precipitation of Warmest Quarter (BIO18) had the top contribution on the BRT model (53.8%) and FDA (48.4%).

Current and future distribution of **Meta** ***spiders in Sicily***

The ensemble models for both species agreed with the known distribution of the two species on Mount Etna (Fig. 4). The current suitable area for *Meta bourneti* was predicted from the sea level up to an altitude of approximately 1100 meters along all the slopes of the volcano (Fig. 4A). The current suitable area for *Meta menardi* was predicted along a narrow strip on Mount Etna, between 1000 and 1600 meters a.s.l.

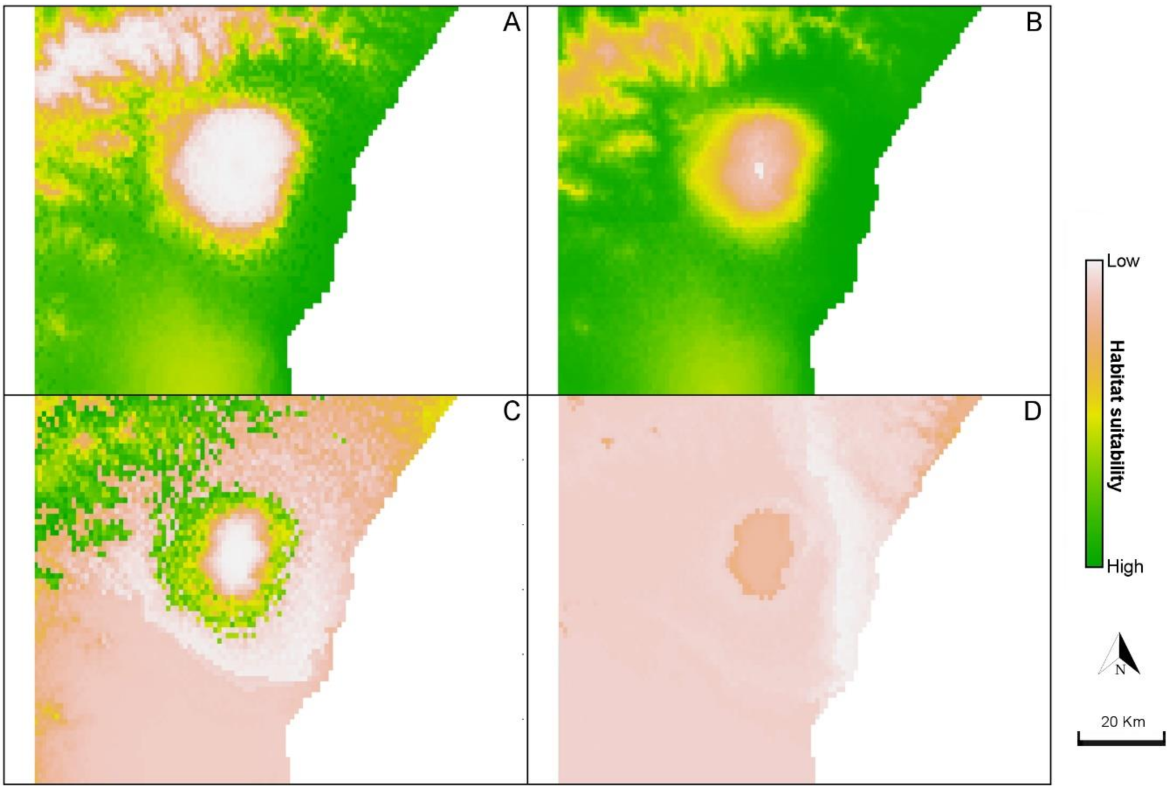

Figure 4. Predicted current (1970-2000) (A) and future (2070) under the high-emission scenario RCP 8.5 (B) distribution for M. bourneti on Mount Etna. Predicted current (C) and future (D) distribution for M. menardi on Mount Etna. All predictions are based on ensemble of GLM, BRT and FDA models.

The averaged future prediction under the RCP 8.5 scenario in the year 2070 showed a gain in habitat suitability for *Meta bourneti* along the upper slopes of Mount Etna. The species is expected to extend its distribution up to approximately 1600 meters a.s.l. on the southeastern side, and at slightly lower elevations on the northern slope of the volcano, interfering with sites that are currently colonized by *Meta menardi* (Fig. 4C). The future predictions for 2070 showed the complete loss of habitat for *M. menardi* on Mount Etna (Fig. 4D). Under the RCP 8.5 scenario in the year 2070 the species will go through a considerable reduction in the area of suitable-habitat areas, as the species will not be able to shift its distribution in the upper areas of Mount Etna.

DISCUSSION

Meta bourneti and *M. menardi* are two of the most frequent species that can be encountered in the twilight zone of caves and other hypogean sites in Europe (Mammola & Isaia, 2017).

The former is widely distributed in the Mediterranean basin (Nentwig et al. 2022) and its ecological preference for warmer hypogean sites (above 9°C) makes it particularly abundant in Southern Europe. Conversely, *M. menardi* shows a preference for cooler hypogean sites (Isaia et al. 2007; Mammola & Isaia 2014).

Mammola et al., 2021 have tested the role of both latitudinal and elevational gradients in determining the global distribution range of *M. bourneti* and *M. menardi*, highlighting a clear example of the trade-off between latitude and elevation in determining habitat segregation of broadly distributed competing species. It follows that it is the microclimate to be one of the most important factors determining the niche segregation in *Meta* spiders, as proved for the Italian (Mammola & Isaia 2014; Mammola 2017; Brignoli 1971) and British population (Mammola, 2017). In the Pyrenean region, *M. bourneti* is preferably found in caves below 900 meters a.s.l., while *M. menardi* is found mainly above 900 m a.s.l. (Ribera, 1978).

The same trend has been recently observed on Mount Etna (Paper V), where sampling activities on lava caves have led to the discovery of several new occurrences of *M. bourneti* and the new record of *M. menardi* for Sicily (Paper V) which gave impulse to this work unrevealing a neat separation between the two species in terms of habitat preference along the altitudinal gradient of Mount Etna. In contrast with the observations at higher latitudes (e.g., Mammola et al., 2020; Mammola et al., 2021), here *M. menardi* occurs exclusively in caves above 1200 meters, whereas *M. bourneti* prefers sites at lower elevations.

In this study, we provided a detailed analysis of the main factors contributing to segregation of the two species. Moreover, we modelled their potential distribution under current and future climatic conditions at local scale in view of the conservation of the Sicialian populations of *M. menardi.*

Mount Etna represents a remarkable biotope in Europe, threatened by both natural and anthropogenic disturbance. Moreover, the area is particularly sensitive to climate change, as migration on mountains of species upwards can occur to only a limited extent (Thuiller, 2007). Morever, Mount Etna represents a very active volcano presenting fast-moving lava flows and frequent effusive eruptions from vents located on the flanks of the volcano that produces lava that may reach long distances (Calvari and Pinkerton, 1998; Del Negro et al., 2016, 2020).

The PCA based on microclimatic variables has suggested a clear segregation between *M. bourneti* and *M. menardi*. Temperature related variables (BIO08) seem to favour the occurrence of *M. bourneti*, but the species seems negatively influenced by precipitation-related variables (BIO19), indicating a preference of the species for warmer and drier sites. On the other side, *M. menardi* colonises sites at higher elevation where temperature is lower (slight contribution of BIO07) and the amount of precipitation is generally higher (notable contribution of BIO18). Similarly, caves presenting higher humidity seem to enhance the presence of *M. menard*i in the Pre-alpine region in Lombardy (Italy) (Manenti at al., 2015). Mammola & Isaia, 2014 reported a conditional differentiation of the niche among M. menardi and M. bourneti in the Italian Wester Alps, with the first being more competitive at narrow ranges of cooler temperature and higher relative humidity and *M. bourneti* limited in a low number of caves climatically unsuitable for *M. menardi.*

In contrast with Mammola & Isaia 2017, which described a significant overlap in their niches in the Italian Wester Alps, the analysis of the niche hypervolume of the two species on Mount Etna has shown a marked difference of their four-dimensional hypervolume (Fig. 3). *Meta menardi* showed a markable smaller bioclimatic niche compared to *M. bourneti*. Moreover, the niche hypervolume of *M. menardi* results almost completely comprised that of M. bourneti. This suggests the ability of both species to exploit a similar variety of microhabitats in subterranean habitat as highlighted in other study areas (Mammola & Isaia, 2014; Mammola & Isaia, 2017; Nentwig et al., 2022; Smithers, 2005), although *M. bourneti* seems to exhibit a higher ecological plasticity in terms of microclimatic requirements. The hypervolume of *M. bourneti* was found to be 45-fold wider than that of *M. menardi*, suggesting that this species is more stenoecious than M. bourneti, as widely documented in other investigations (Mammola & Isaia, 2014; Mammola & Isaia, 2017; Novak et al., 2010).

In light of its higher ecological plasticity *M. bourneti* seems able to colonise a greater diversity of environments, including caves that open in forest, agricultural and urban areas. As for *M. menardi*, the species was mainly found in vegetated sites (i.e. forests).

As matter of fact, neither of the species seems able to colonise volcanic caves of Mount Etna over 1,600 meters, probably due to unfavourable microclimatic conditions or, more likely, to the lack of suitable habitat. These mainly represent areas of recent lava fields devoid of vegetation due to the major frequency of volcanic events (i.e., volcanic ash fall and presence of lava flows).

A higher number of volcanic events in the summit areas prevents the establishment of the vegetation, which generally requires over 20 years for the first cryptogam stage or any other colonisation stage (Poli & Grillo, 2000). Considering the key role of vegetation in facilitating the dispersion of the epigean juvenile stages (Smithers, 2005), these conditions are likely to limit the colonisation. This is reasonable when considering that *M. menardi* has been observed in caves up to 2,000 m a.s.l. opening in vegetated areas (pastures) (Isaia et al. 2011; Mammola et al. 2021) and that most sites opened in recent lava fields are not colonised by both species, although potentially suitable for the species from a climactic point of view.

The suitable areas predicted by the models for both species agreed with its current range distribution on Mount Etna (Fig. 4A, C). For *M. bourneti*, the current suitable habitats were predicted along all the slopes of the volcano, from the sea level up to 1100 meters, from where *M. menardi starts to be present.* For the latter, the Mean Temperature of Wettest Quarter (BIO08) emerged as the main driver of distribution, in accordance with the low tolerance to lower temperatures mentioned in Mammola & Isaia, 2014 and Manenti et al., 2015.

For *M. menardi*, the present distribution was predicted along a narrow strip on Mount Etna, between the areas colonised by *M. bourneti*, and those at higher altitudes interested by in recent volcanic activity. A notable contribution of the Precipitation of Warmest Quarter (BIO18) and a slight contribution of the Temperature Annual Range (BIO07) were detected when considering the habitat suitability of *M. menardi*, indicating its preference for humid areas, especially in the warmer months, and its low tolerance to higher temperature variations, as widely documented for this species (Mammola & Isaia, 2014; Manenti et al., 2015).

The averaged future predictions from the three climatic models for 2070 showed that both species will be affected by climate change (Fig. 4B, D). *Meta bourneti* is likey to be favoured by the increase of temperature and expand its distribution toward new suitable areas up to 1,300 meters possibly colonizing caves currently occupied by *M. menardi*.

At the same time, predictions for M. menardi would potentially lead to the abandonment of southern caves on Mount Etna, causing a significant loss of suitable areas, likely determining the disappearance of the species from the study area (Fig. 4D).

If, from one hand, the species will shift its distribution towards the upper parts of the volcano, encountering those sites unsuitable from both species due to the presence of young substrate devoid of vegetation, on the other, the species will face the competition with M. bourneti, favoured by the rise of temperature due to the climate change.

Considering that both species generally colonise the outermost sectors of caves (Mammola et al., 2016), they will likely react to climatic change in a relatively short time-scale (Mammola & Isaia, 2017). Interestingly, in 2021, we were able to detect the replacement of *M. menardi* by *M. bourneti* in “Grotta di Piano Noce” (1195 m a.s.l), a lava cave located approximately at a linear distance of 1 km from “Grotta di Piano Porcaria” (1099 m a.s.l), the sites where the two species get previously closer.

Despite being an isolated event, it seems likely that the replacement of *M. menardi* by *M. bourneti*, and the northward expansion of both species, will occur in the short term. However, the potential expansion in the summit areas of the volcano will be limited by the environmental barrier represented by the young lava substrate, which may represent a hostile environment for the epigean juvenile phase of *M. menardi* (Paper V). It is in fact unlikely that the vegetation will shift its distribution in the upper section of the volcano frequently affected by the volcano activities, due to the rather long colonisation times of the vegetation (Poli & Grillo, 2000).

Considering that local populations of many species cannot shift their climatic niches rapidly enough to prevent extinction (Román-Palacios & Wiens, 2020), and the difficulty of planning prevention strategies to reduce impacts of global climate change on a national and global scale (Prabhakar, 2008), investigations and monitoring activities at the local level would be necessary to establish an appropriate management plan for the conservation of the population of *M. menardi* in Sicily.

ACKNOWLEDGEMENT

We thanks the “Ente Parco dell’Etna” that authorized and allowed the carrying out of the scientific research activity in the Park of Etna.

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SUPPLEMENTARY MATERIALS

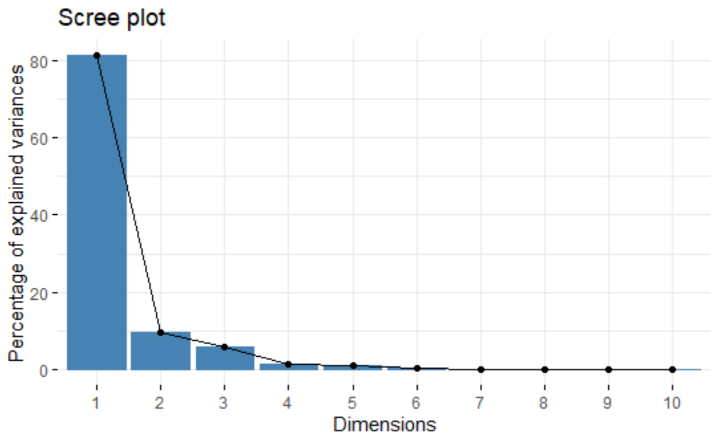

Figure S1. scree plot of the PCA displaying the percentage of explained variance for the first 10 axes.

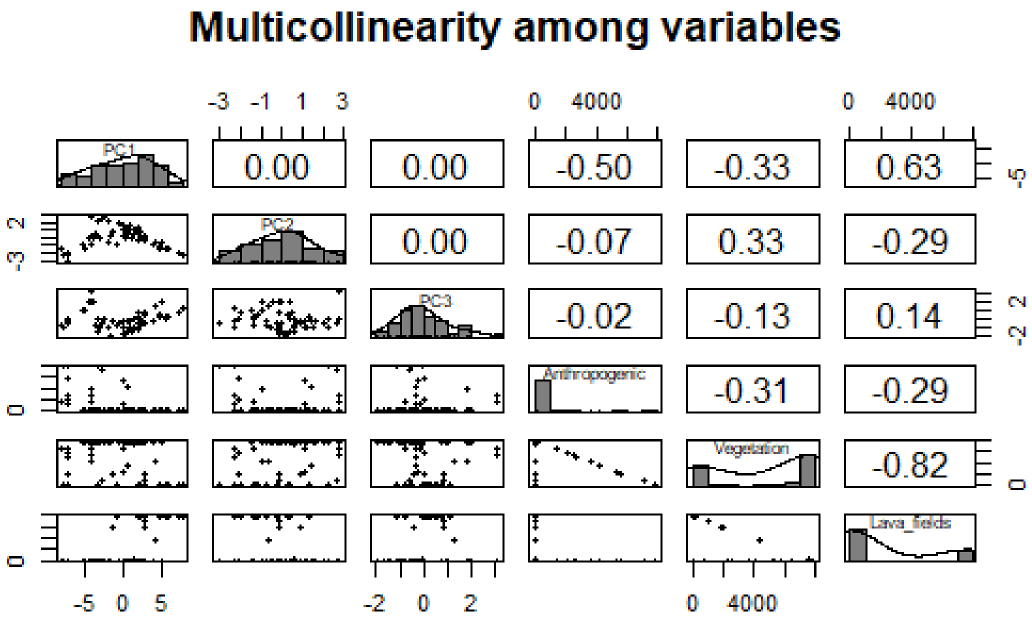

Figure S2. Results of the correlation test among PCA axes summarizing bioclimatic variables (PC1, PC2 and PC3) and land use variables (Anthropogenic, Vegetation and Lava fields) with values of the Pearson's r on the upper right and plots of the values on the bottom left. The diagonal plots represent histograms of the analysed variables.

Table S1. Bioclimatic variables used in this study.

Variable	**Description**	**Unit**
BIO01	Annual Mean Temperature	°C
BIO02	Mean Diurnal Range (Mean of monthly (max temp–min temp))	°C
BIO03	Isothermality (BIO2/BIO7) (×100)	°C
BIO04	Temperature Seasonality (standard deviation ×100)	°C
BIO05	Max Temperature of Warmest Month	°C
BIO06	Min Temperature of Coldest Month	°C
BIO07	Temperature Annual Range (BIO5-BIO6)	°C
BIO08	Mean Temperature of Wettest Quarter	°C
BIO09	Mean Temperature of Driest Quarter	°C
BIO10	Mean Temperature of Warmest Quarter	°C
BIO11	Mean Temperature of Coldest Quarter	°C
BIO12	Annual Precipitation	mm
BIO13	Precipitation of Wettest Month	mm
BIO14	Precipitation of Driest Month	mm
BIO15	Precipitation Seasonality (Coefficient of Variation)	mm
BIO16	Precipitation of Wettest Quarter	mm
BIO17	Precipitation of Driest Quarter	mm
BIO18	Precipitation of Warmest Quarter	mm
BIO19	Precipitation of Coldest Quarter	mm

Paper VII

BIOLOGICAL
REVIEWS

Cambridge
Philosophical Society

Biol. Rev. (2023), pp. 000–000.
doi: 10.1111/brv.12933

Aliens in caves: the global dimension of biological invasions in subterranean ecosystems

Giuseppe Nicolosi[1†], Stefano Mammola[2,3†], Laura Verbrugge[4] and
Marco Isaia[1,*]

[1]*Department of Life Sciences and Systems Biology, University of Turin, Via Accademia Albertina 13, 10123, Torino, Italy*
[2]*Molecular Ecology Group (Dark-MEG), Water Research Institute (IRSA), National Research Council (CNR), Corso Tonolli, 50, Pallanza, 28922, Italy*
[3]*Laboratory for Integrative Biodiversity Research (LIBRe), Finnish Museum of Natural History (LUOMUS), University of Helsinki, Pohjoinen Rautatiekatu 13, Helsinki, 00100, Finland*
[4]*Water and Development Research Group, Department of Built Environment, Aalto University, Tietotie 1E, Espoo, 02150, Finland*

ABSTRACT

Alien species are a significant threat to natural ecosystems and human economies. Despite global efforts to address this challenge, the documented number of alien species is rapidly increasing worldwide. However, the magnitude of the impact of alien species may vary significantly across habitats. For example, some habitats are naturally less prone to biological invasions due to stringent abiotic and biotic characteristics, selecting for a limited number of introduced species possessing traits closely related to the native organisms. Subterranean ecosystems are quintessential examples of habitats with strong environmental filters (e.g. lack of light and scarcity of food), driving convergent adaptations in species that have successfully adapted to life in darkness. Despite these stringent environmental constraints, the number of records of alien species in subterranean ecosystems has increased in recent decades, but the relevant literature remains largely fragmented and mostly anecdotal. Therefore, even though caves are generally considered very fragile ecosystems, their susceptibility to impacts by alien species remains untested other than for some very specific cases. We provide the first systematic literature survey to synthesise available knowledge on alien species in subterranean ecosystems globally. This review is supported by a database summarising the available literature, aiming to identify gaps in the distribution and spread of alien invertebrate species in subterranean habitats, and laying the foundations for future management practices and interventions. First, we quantitatively assessed the current knowledge of alien species in subterranean ecosystems to shed light on broader questions about taxonomic biases, geographical patterns, modes of dispersal, pathways for introductions and potential impacts. Secondly, we collected species-specific traits for each recorded alien species and tested whether subterranean habitats act as ecological filters for their establishment, favouring organisms with pre-adaptive traits suitable for subterranean life. We found information on the presence of 246 subterranean alien species belonging to 18 different classes. The dominant alien species were invertebrates, especially insects and arachnids. Most species were reported in terrestrial subterranean habitats from all continents except Antarctica. Palaearctic and Nearctic biogeographic regions represented the main source of alien species. The main routes of introductions into the recipient country are linked to commercial activities (84.3% of cases for which there was information available). Negative impacts have been documented for a small number of case studies (22.7%), mostly related to increased competition with native species. For a limited number of case studies (6.1%), management strategies were reported but the effectiveness of these interventions has rarely been quantified. Accordingly, information on costs is very limited. Approximately half of the species in our database can be considered established in subterranean habitats. According to our results, the presence of

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[*] Author for correspondence (Tel.: +00390116704544; E-mail: marco.isaia@unito.it).

[†]Equal first authors.

suitable traits grants access to the stringent environmental filter posed by subterranean environments, facilitating establishment in the new habitat. We recommend that future studies deepen the understanding of invasiveness into subterranean habitats, raising public and scientific community awareness of preserving these fragile ecosystems.

Key words: subterranean biology, conservation biology, invasiveness, subterranean alien species, adaptive traits, allochthonous species.

CONTENTS

I. INTRODUCTION

In a globalised planet, there has been an increase in human-mediated relocations of species beyond their natural ranges (Meyerson & Mooney, 2007; Hulme *et al.*, 2008; Liebhold & Tobin, 2008). Alien species are defined as organisms introduced accidentally or deliberately into a habitat where they are not normally found, often representing a serious threat to biodiversity and the functioning of ecosystems (Pyšek *et al.*, 2020; Clavero & García-Berthou, 2005; Simberloff *et al.*, 2013). In recent years, the number of successful biological invasions has continued to rise, despite increasing global conservation efforts to address this challenge (Pagad *et al.*, 2015), often resulting in substantial impacts to ecosystems (Vilà *et al.*, 2010, 2011) and economies (the global cost of invasive alien species is estimated to be a minimum of $26.8 billion annually; Diagne *et al.*, 2021).

With increasing study of the potential impacts of alien species across taxonomic groups and habitat types (e.g. Courchamp *et al.*, 2017; Cuthbert *et al.*, 2019; Haubrock *et al.*, 2019; Mofu *et al.*, 2019), there is a growing awareness that not all natural environments are equally likely to be invaded (Pyšek, Chytrý & Jarošík, 2009; Pyšek *et al.*, 2010). Due to their abiotic and biotic characteristics, some habitats may be less prone to biological invasions than others. As foreseen by Charles Darwin, preadaptation and competition are the two key opposing forces behind the success or failure of an invasion (Cadotte *et al.*, 2018). In other words, when a habitat exerts a strong environmental filter, colonisers showing traits that are closely related to local native organisms may be more successful than others. Conversely, when competition is the most important factor shaping a community, selection will act against trait similarity and colonisers with comparable traits are generally excluded – the so-called 'Darwin's naturalisation hypothesis'. As a consequence, an enhanced understanding of community assembly rules in a functional perspective is crucial to assessing invasion risks (Hamilton *et al.*, 2005; Statzner, Bonada & Dolédec, 2008; Cadotte *et al.*, 2018).

Caves and other subterranean systems are quintessential examples of habitats with strong environmental filters, selecting for convergent adaptations in species that have successfully adapted to life in darkness (Pipan & Culver, 2012; Trontelj, Blejec & Fišer, 2012). Eye reduction, depigmentation and enhanced development of tactile and olfactory organs are among the most conspicuous features possessed by subterranean species; these shared features have evolved in response to selective environmental pressures imposed by subterranean environments. As a result, one can predict that the conditions in deep subterranean habitats should act as effective ecological filters for the establishment of alien species, favouring only those organisms with suitable pre-adaptive traits (Reeves, 1999; Mammola, 2017). Alien species can successfully establish in surface/subterranean ecotones such as cave entrances and other shallow subterranean spaces due to their higher availability of resources and greater richness and diversity of native species (Lloyd *et al.*, 2000; Prous, Ferreira & Martins, 2004; Prous, Ferreira & Jacobi, 2015).

Despite an increased number of records of alien species in subterranean ecosystems during recent decades, relevant literature remains scarce and fragmented. Moreover, occurrences mostly refer to caves or to artificial hypogean habitats (i.e. bunkers and abandoned mines), with few studies on other kinds of – still largely unexplored – subterranean habitats (e.g. the *Milieu Souterrain Superficiel*; see Mammola *et al.*, 2016). For these reasons, the true extent of alien species invasions in the subterranean realm is largely unknown and in-depth studies are needed to clarify the importance of this threat in terms of biological conservation and how best to address any related environmental issues. Consequently, assessing the effects of alien species on subterranean ecosystems is perceived as an important and urgent question in cave-based science (Mammola *et al.*, 2020).

To facilitate this goal, we here provide a first synthesis of the available literature on alien species in subterranean ecosystems. We asked three general questions: (*i*) hat are the most frequent alien taxa present in subterranean habitats? (*ii*) What are the origins, the recipient countries, and the pathways of alien species introductions in subterranean ecosystems? (*iii*) What are the environmental and socio-economic impacts of these species? We then extracted information on species-specific traits for each alien species documented in subterranean ecosystems across the sampled literature, aiming to answer a further question: (*iv*) do successful colonisers of subterranean environments display pre-adaptive traits? Specifically, we tested the relationship between the presence/absence of adaptive traits facilitating the colonisation of subterranean ecosystems and the probability of establishment in a subterranean environment. Considering that the strength of competition in caves is often lower than that of environmental filtering (Mammola, 2019), we predict that successful colonisers should possess traits that are similar to those of local native organisms (Cadotte *et al.*, 2019).

II. MATERIALS AND METHODS

(1) Scope of the analysis

We focused on terrestrial and freshwater subterranean habitats globally. Following the function-based classification of Earth's ecosystems (Keith *et al.*, 2022), the habitats we considered were 'Subterranean' (S) [including 'Subterranean lithic' (S1) and 'Anthropogenic subterranean voids' (S2) biomes] and 'Subterranean-freshwater' (SF) [including 'Subterranean freshwater' (SF1) and 'Anthropogenic subterranean freshwater' (SF2) biomes]. We excluded marine caves and anchialine systems, i.e. the 'Subterranean tidal' (SM1) biome *sensu* Keith *et al.* (2022). The diversity of alien species, pathways of introduction, and management in marine systems seems to be much lower than in terrestrial ecosystems [see Gerovasileiou *et al.* (2016, 2022) for extensive coverage of alien species in marine caves].

Furthermore, we did not consider studies focusing on alien photosynthetic organisms (lampenflora) in caves opened to tourism (i.e. illuminated by artificial lights; see e.g. Cigna, 2011; Falasco *et al.*, 2014; Mulec, 2019; Piano *et al.*, 2015; Piano, Nicolosi & Isaia, 2021). We excluded studies on lampenflora because the species pool of photosynthetic organisms colonising a cave usually originates from the surface habitat in the proximity of the cave rather than a different biogeographic region. In addition, this topic has been the subject of other reviews (Baquedano Estévez *et al.*, 2019; Falasco *et al.*, 2014; Piano *et al.*, 2022).

We considered alien subterranean species to be alien species moved by human activities beyond the limits of their natural geographic range into a new area (*sensu* Richardson, Pyšek & Carlton, 2011) and invading any of the subterranean systems considered herein (i.e. S1, S2, SF1 and SF2, see above). We acknowledge that this is a broad generalisation: nativeness is a nuanced and highly dynamic concept (Lemoine & Svenning, 2022; Verbrugge, Leuven & Zwart, 2016) whose assessment necessarily entails a certain degree of interpretation and subjectivity. However, given the scarce information available, we found this simplification to be appropriate for our analysis.

(2) Standardised literature search

We conducted a systematic literature review focused on the occurrence of subterranean alien species. In August 2021, we performed standardised literature searches in the Clarivate Analytics *Web of Science*. For the literature search, we followed the PRISMA reporting standard (Moher *et al.*, 2009; O'Dea *et al.*, 2021).

We initially trialled several combinations of words for our *Web of Science* query string, aiming to improve the search specificity. During this exploratory trial, we found that the use of generic terms such as 'Subterranean' and 'cave' resulted in an excess of irrelevant articles often referring to archaeological, anthropological, or mineralogical aspects. To minimise this number of irrelevant references, we added a 'NOT' criterion while also restricting our search to *Web of Science* Categories referring to natural science and biology. The final search string was: TS = ("cave*" OR "hypoge*" OR "subterranean" OR "lava tube*") AND TS = ("alien*" OR "invasive" OR "introduced" OR "exotic" OR "non-native" OR "non native" OR "non-indigenous" OR "non indigenous") NOT TS = ("termite*" OR "fungi" OR "marine" OR "architecture" OR "Archaeol*" OR "microbial" OR "medicine" OR "speleogenesis" OR "art" OR "histor*" OR "agricult*") AND WC = (Ecology, Zoology OR Entomology OR Geosciences Multidisciplinary OR Biodiversity Conservation OR Multidisciplinary Sciences OR Agriculture Multidisciplinary OR Environmental Sciences OR Plant Sciences OR Geology OR Agronomy OR Marine Freshwater Biology OR Biology OR Genetics Heredity OR Soil Science OR Biotechnology Applied Microbiology OR Forestry OR Evolutionary Biology OR Education Educational Research OR Fisheries OR Horticulture OR Microbiology OR Veterinary Sciences OR Agriculture Dairy Animal Science OR Oceanography OR Toxicology OR Anatomy Morphology OR Mycology OR Education Scientific Disciplines OR Infectious Diseases OR Ornithology).

This initial search yielded 2781 papers. We screened the titles and abstracts of all papers obtained from this search for eligibility to be included in the review, selecting $N = 448$ for potential inclusion. We then read the full text of each of these papers to select relevant studies based on a set of inclusion/exclusion criteria. We included studies if they: (*i*) investigated the state of subterranean ecosystem components potentially impacted by alien species; (*ii*) provided subterranean fauna inventories including the presence of subterranean alien species; and (*iii*) investigated the effect of management practices in subterranean ecosystems to control or eradicate subterranean alien species. We excluded studies that: (*iv*) focused on subterranean alien species in non-subterranean habitats; (*v*) focused on 'Subterranean tidal' ecosystems (SM1, see Section II.1). A total of 43 papers met our criteria (Fig. S1).

We cross-checked the resulting list of subterranean alien species with the Global Biodiversity Information Facility (GBIF; www.gbif.org; accessed December 2021) and International Union for Conservation of Nature (IUCN) ISSG Global Invasive Species Database (www.iucngisd.org/gisd/; accessed December 2021) to verify the current status (i.e. if the species is currently considered as alien in the specific country) and level of invasiveness of the alien species present in our database.

(3) Additional literature search

Given that the literature on alien species includes grey literature not listed on the *Web of Science*, including technical reports and articles not in English (Haddaway *et al*., 2020; Chowdhury *et al*., 2022), we conducted parallel searches for additional papers to maximise the comprehensiveness of our database. For each paper selected above, we inspected the reference list to retrieve additional potentially relevant literature. We also performed a search in *Google Scholar* (Haddaway *et al*., 2015) using the same key words listed in Section II.2. These additional searches resulted in 61 papers added to our database (Fig. S1).

(4) Meta-data extraction

The full list of the metadata extracted and their sources is presented in Table 1. The literature database is provided as online supporting information in Table S1. For each paper, we read the full text and extracted detailed information (Fig. 1), including the year of the study and the country where the study occurred. Next, we recorded the alien species (see definition in Section II.1) mentioned in each publication, its most recent taxonomy (based on the GBIF database), the domain (terrestrial or freshwater), and the type of subterranean habitat in which the species was found using Keith *et al*. (2022) as: 'Subterranean lithic', 'Anthropogenic subterranean voids', 'Subterranean freshwater', or 'Anthropogenic subterranean freshwater'. In addition, we subdivided the subterranean lithic habitat into 'limestone cave' and 'lava tubes'.

We included the biogeographic region of origin of the alien species (Global, Afrotropical, Indomalayan, Nearctic, Neotropical, Oceanian, Palaearctic, Unknown), based on the information reported complemented by species-specific literature searches.

We included a generic indication of the possible establishment of the subterranean alien species (Occasional, Naturalised, Unknown) based on the information provided in each paper. If not specifically stated, we considered as naturalised (i.e. established) a species forming plausible self-replacing populations (i.e. abundant, spread across multiple locations and present throughout the year) (Richardson *et al*., 2000). In other cases, we considered the species as 'Occasional'. When the information was missing or insufficient to define its status, we classified it as 'Unknown'.

For the type of impact, impact outcome, and management activities we referred to the categories/classifications present in the IUCN Global Invasive Species Database.

Based on the information reported in each publication, we registered the impact outcome of the subterranean alien species (Ecosystem/habitat, Species/population, and/or Socio-economic, or Unknown), and performed an overall assessment of the direction of this impact (Positive, Negative, Neutral or Unknown). For Socio-economic impact, we also used the InvaCost database (version 4.0) (Diagne *et al*., 2020*a*,*b*) to obtain an estimate of the globally reported costs of that alien species. Although the available data do not specifically refer to subterranean habitats, they provide a proxy indication of the potential socio-economic impact in subterranean habitats.

We classified the ecological impacts on the subterranean habitat caused by each species into 13 mechanisms: Competition (the alien species competes with cave-dwelling native taxa for resources); Predation (the alien species predates cave-dwelling native taxa); Hybridisation (the alien species hybridises with cave-dwelling native taxa); Disease transmission (the alien species transmits diseases to native cave-dwelling species); Parasitism (the alien taxon parasitises cave-dwelling native taxa); Poisoning/toxicity (the alien taxon is toxic or allergenic to cave-dwelling native taxa); Bio-fouling (the alien taxon deposits on surfaces or septa of cave-dwelling native taxa, compromising their functionality); Grazing/herbivory/browsing (the alien species affects the functional species composition of plant communities);

Table 1. Summary of the extracted metadata and their sources.

Metadata	Sources	Description	Levels
Species	Investigated literature	Scientific name of the subterranean alien species	–
Class	GBIF backbone taxonomy	Class of the subterranean alien species	–
Order	GBIF backbone taxonomy	Order of the subterranean alien species	–
Organism group	Investigated literature	Taxonomic group included in the database	Invertebrate; Vertebrate; Plant
Trophic level	Investigated literature	Level or position in food chain, food web, or ecological pyramid of the subterranean alien species	Detritivore; Herbivore; Omnivore; Parasite; Predator; Primary producer
Location	Investigated literature	Invaded country out of the native range of the subterranean alien species	–
Domain	Investigated literature; General literature	Type of ecosystem in which the subterranean alien species occurs	Terrestrial; Freshwater
Microhabitat	Investigated literature	Type of habitat in which the subterranean alien species occurs	Subterranean lithic; Anthropogenic subterranean voids; Subterranean freshwater; Anthropogenic subterranean freshwater
Origin continent	Investigated literature; GISD; GBIF	Continent in which the subterranean alien species originated and/or where it first arrived without human intervention. Species with a 'Cosmopolitan' distribution are recognised as alien although their specific geographic origin is unknown.	Asia; Africa; North America; South America; Antarctica; Europe; Oceania; Unknown; Cosmopolitan
Biogeographic origin	Literature; General literature; GISD; GBIF	Bioregion in which the subterranean alien species originated and/or where it first arrived without human intervention	Global; Afrotropical; Indomalayan; Nearctic; Neotropical; Oceanian; Palaearctic; Unknown
Established	Investigated literature	Indication of the possible naturalisation of the subterranean alien species into the new habitat/country	Naturalised; Occasional; Unknown
Adaptive trait	Investigated literature; General literature	Indication of the presence or absence of adaptations commonly present in the subterranean alien species	Yes; No
Trait	Investigated literature; General literature	Type of adaptation present in the subterranean alien species	Absence of eyes; Behavioural traits; Depigmentation; Elongated appendages; Eyes reduction; Physiological adaptations
Presence of wings	Investigated literature; General literature	Considered a proxy for dispersal ability	Yes; No
Impact	Investigated literature	General impact caused by the subterranean alien species	Positive; Negative; Neutral; Unknown
Mechanism	Investigated literature	Any change in ecological or ecosystem properties, excluding socio-economic effects and human values	Competition; Predation; Hybridisation; Disease transmission; Parasitism; Poisoning/toxicity; Bio-fouling; Grazing/herbivory/browsing; Rooting/digging; Trampling; Flammability; Interaction with other invasive species; Other; Unknown
Impact outcome	Investigated literature; InvaCost database (for socio-economic impacts only)	Impact of subterranean alien species: changes to environmental or socio-economic parameters	Ecosystem – Habitat; Species – population; Socio-economic; Unknown
Pathway	Investigated literature; General literature; GISD; GBIF	Pathways of introduction: how a species is transported (intentionally or unintentionally) outside its natural geographical range	Release; Escape; Transport – contaminant; Transport – stowaway; Corridors; Unaided; Unknown

(Continues on next page)

Table 1. *(Cont.)*

Metadata	Sources	Description	Levels
Management	Investigated literature	Any lethal or non-lethal action aimed at the eradication, population control or containment of a population of an invasive alien species	Prevention; Eradication; Control; Monitoring; None

In 'Sources', 'Investigated literature' refers to the literature extracted in our systematic survey; 'General literature' refers to additional literature sourced for each species using *Google Scholar* and by inspecting reference lists.
GBIF, Global Biodiversity Information Facility; GISD, Global Invasive Species Database.

Rooting/digging (the alien species alters the soil layers); Trampling (the alien taxon causes impacts on substrate properties); Flammability (the alien species modifies the fire regime by altering the inherent flammability of the ecosystem); Interaction with other invasive species (the alien species interacts with other introduced species); Other (other impacts not included above); and Unknown (no documented impact). Note that a single species may fit into multiple categories.

We noted management activities (either suggested or implemented) to prevent or limit the spread of the alien species: Prevention (any measures aimed at preventing alien species from entering a nation or habitat); Eradication (any practice that aims to eradicate the alien species completely); Control (any long-term practice for limiting abundance or density of the alien species); Monitoring (any short- or long-term monitoring program of the status of an alien species); and None (no actions in place, or none known, to prevent the presence or spread of the alien species).

We specified the pathway through which the species reached the recipient region according to the Convention on Biological Diversity (CBD) pathway categorisation (CBD, 2014). Pathways included seven categories: Release (released intentionally for the purpose of human activities, e.g. biological control, fishery, hunting activities, or others); Escape (released unintentionally from confinement, e.g. aquaria, aquaculture, or scientific research); Transport – contaminant (the alien species has a trophic or biotic relationship to organisms or items being transported and on which its survival depends); Transport – stowaway (the alien species has no trophic or biotic relationship to the organisms or items being transported or, if there is any, the alien can survive in their absence); Corridors (dispersed through the establishment of an anthropogenic dispersal corridor such as tunnels or bridges); Unaided (moved naturally across borders); and Unknown (unknown pathway). When available, we also specified the pathway by which alien species were introduced into new subterranean environments within a recipient region, following the same categorisation (see pathways in bold in Table S1).

(5) Species-level traits

We referred to specialised literature to collect species-specific traits for each subterranean species in our database. In the absence of universal criteria that could be applied to quantify the degree of subterranean adaptation, we reported the presence/absence of adaptations commonly present in subterranean species (Pipan & Culver, 2012) based on the biological information available for each species. We scored the following traits: Depigmentation, Absence of eyes, Eyes reduction, Elongated appendages, Behavioural traits, and Physiological adaptations (e.g. lower metabolic rate, reduction in the number of eggs, increased longevity). We also recorded the presence/absence of wings as a proxy for dispersal ability (presence of wings). We also collected data on the trophic level of the subterranean alien species (Detritivore, Herbivore, Omnivore, Parasite, Predator, Primary producer) based on the biological information available for each species.

(6) Data analysis

We carried out analyses in R version 4.2.0. (R Core Team, 2021). We summarised data on alien species in subterranean ecosystems using bar charts and other graphical tools from the package *ggplot2* version 3.3.6. (Wickham, 2016). We visualised the geographic dimension of biological invasions in subterranean ecosystems by projecting onto a global map a network connecting the biogeographic region of origin and the recipient country for each species included in the database.

Finally, we constructed a regression model to explore the role of species traits in explaining the probability of a given alien species establishing in subterranean habitats (see bottom panel in Fig. 1). For model construction and validation, we followed Zuur & Ieno (2016). Given that the response variable is binary (species is established or not) we modelled data using a Bernoulli distribution and a cloglog link function, suitable for an unbalanced binary distribution in the response variable. We fitted the model using a generalised linear mixed model (GLMM) with the R package *lme4* version 1.1–27 (Bates *et al.*, 2015). The structure of the model, in R notation, was: y ∼ Adaptive traits + Trophic level + Presence of wings + (1 | Class/Order), where: 'Adaptive traits' is the presence or absence of any adaptive trait related to subterranean life (see Section II.5 and Table 1 for the full list), which we interpreted as possible preadaptations aiding alien species to overcome the environmental filter posed by subterranean environments (Mammola, 2017). We only considered the explanatory variable 'Adaptive traits' in our model rather than each single trait given the limited number of species

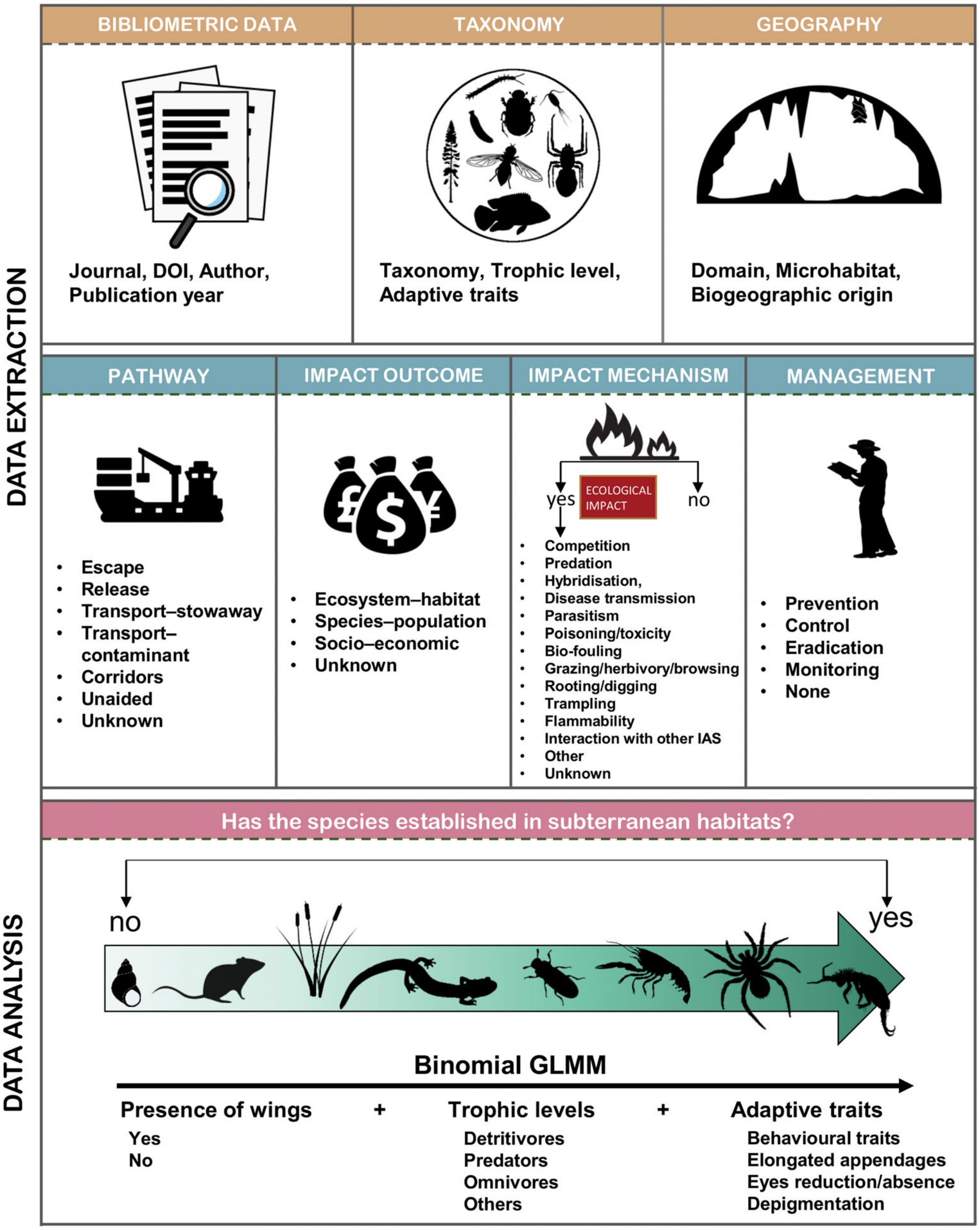

Fig. 1. Infographic summarising the study workflow (data extraction and data analysis). See Section II.6 for variables included in the analysis. GLMM, generalised linear mixed model; IAS, invasive alien species.

presenting subterranean traits, and the consequent prevalence of zeros (i.e. absence of traits).

'Trophic level' is a categorical variable that we included to test whether different trophic groups are more likely than others to establish in subterranean habitats. We used the trophic levels Detritivore, Predator, Omnivore, and Others; with 'Others' here including the least common trophic levels Herbivore, Primary producer and Parasite, which we grouped together to balance factor levels. 'Presence of wings' refers to the presence or absence of wings, which we interpreted as a proxy for dispersibility in a range-expanding population. The random structure of the model was used to control for data non-independence, under the assumption that taxonomically related species may express more similar traits than expected from random. We validated the model with the R package *performance* version 0.9.0. (Lüdecke *et al.*, 2021).

III. RESULTS AND DISCUSSION

We included 104 publications in the final database (Table S1). Most of these papers were published after the year 2000 (Fig. S2). This body of literature encompasses 362 reports of alien species in subterranean habitats corresponding to 246 unique alien species from 18 classes invading subterranean habitats (Fig. 2).

(1) What are the most frequent alien taxa present in subterranean environments and habitats?

Most of the subterranean alien species were reported in terrestrial subterranean habitats (322/362 cases; 88.9%), rather than freshwater (40/362; 11.0%). Subterranean lithic was the most invaded terrestrial ecosystem (323 cases) of which 194 (53.6%) reports concerned limestone caves and 129 (35.6%) lava caves, followed by anthropogenic subterranean voids with 14 cases (3.87%).

From these 362 cases, we extracted information on 246 unique species invading subterranean habitats. These were mostly invertebrates (211; 85.8%), followed by vertebrates (20; 8.1%) and plants (15; 6.1%) (Fig. 3A). Among invertebrates, arthropods dominated, especially the class Insecta (59 species; 24.0%) followed by Arachnida (46; 18.7%), Entognatha (32; 13.0%), and Diplopoda (19; 7.7%). Vertebrates were represented by Actinopterygii (10 species; 4.1%), followed by Amphibia and Mammalia with five species each (2.0%). This pattern reflects the dominant groups in subterranean food webs (Deharveng & Bedos, 2018). In surface ecosystems insects are considered among the most invasive organisms (Seebens *et al.*, 2017), although current knowledge in invasion ecology might be taxonomically and/or geographically biased (Pyšek *et al.*, 2008). Among invertebrates, the other dominant group was the class Malacostraca (Gastropoda) (22; 8.9%) (Fig. 3A). Despite the general adverse conditions in caves for plants, the class Magnoliopsida constituted 5.3% of all species in our database, being mostly represented by species colonising the entrance zone, or penetrating the soil and reaching the cave with their roots.

Araneae and Collembola were the most dominant orders, represented respectively by 31 species (12.6%) and 30 species (12.2%), followed by Coleoptera (16; 6.5%), Isopoda (14; 5.7%), and Hymenoptera (10; 4.1%) (Fig. 3B). Among vertebrates, Caudata and Cyprinodontiformes were the best represented orders with five species each (2.0%).

The five species identified most often in caves are the diplopod *Oxidus gracilis* (15 cases), followed by the fire-ant *Solenopsis invicta* (14), the cockroach *Periplaneta americana* (7), the spiders *Nesticella mogera* (7) and *Psilochorus simoni* (7) and the worm *Bimastos rubidus* (5).

Subterranean ecosystems are generally regarded as nutrient-poor environments that mainly depend on energy inputs from the surface (Culver & Pipan, 2019). Consequently, food webs are bottom-truncated (Gibert & Deharveng, 2002) and detritus-based; herbivores are usually absent, although cave root feeders may be present (Howarth, 1983). As expected, detritivores were the dominant feeding group among the 246 subterranean alien species detected in subterranean ecosystems, encompassing 81 species (33.0%), followed by predators (70; 28.5%), omnivores (60; 24.4%), and herbivores (18; 7.3%).

(2) What are the origins, recipient countries and pathways of alien species introductions in subterranean ecosystems?

The greatest proportion of alien species in our database has a Palaearctic origin (116; 47.2%), followed by Neotropical (26; 10.6%), Indomalayan (23; 9.3%), Afrotropical (15; 6.1%), Nearctic (18; 7.3%) and Oceanian (10; 4.1%); 15 species (6.1%) have a global distribution, with information lacking for 23 species (9.3%) (Fig. 4).

Palearctic and Nearctic biogeographic regions represent the main source of alien species, with broad bi-directional exchanges between these two regions (Fig. 4). This trend is likely due to the greater economic development of these regions and their associated international trade and globalisation networks (Turbelin, Malamud & Francis, 2017), although could be also attributed to higher research effort on alien species in these regions (Pyšek *et al.*, 2008). In Europe, a broad contingent of species also comes from the Afrotropical and Indomalayan biogeographic regions (Fig. 4).

Records of subterranean alien species spanned 60 countries. The majority were reported from the USA, of which 56.1% are in the Hawaiian Islands (Fig. 4). This high percentage is probably due to extensive efforts by local researchers documenting the alien fauna of Hawaiian lava tubes over several decades (e.g. Howarth, 1978; Howarth *et al.*, 2007; Howarth & Stone, 2020).

Australia, a region with a long history of biological invasions (Bradshaw *et al.*, 2021), had the second highest number of reported subterranean alien species, followed by Spain (of which 88.5% of records were in the Canary Islands) and Italy (Fig. 4). This distribution again may reflect greater research efforts in these countries, as well as the paucity of information on the distribution of alien species in subterranean habitats in most countries. However, these data are in line with the global trend for invasive alien species observed by Turbelin *et al.* (2017).

Although research efforts to understand pathways of biological invasions have increased recently (Meyerson & Mooney, 2007), information on subterranean species is scarce. We could retrieve information on the routes by which alien species were introduced into the recipient country for only 64 out of 362 cases (17.7%). Of these, only in a limited number of cases (18 out of 64, 28.1%) was information about the pathways of introduction into the subterranean habitat specified. The most widespread form of introduction into the recipient country is related to trade activities (54 out of 64 cases, 84.3%: Transport – contaminant, 31 cases, 48.4%; Transport – stowaway, 36 species, 35.9%), especially for invertebrates (Fig. 5A), and in particular for predators and omnivores (Table S1).

The trade in potted plants is possibly the main vehicle of introduction of alien species into subterranean ecosystems. Invertebrate species can be passively dispersed within the

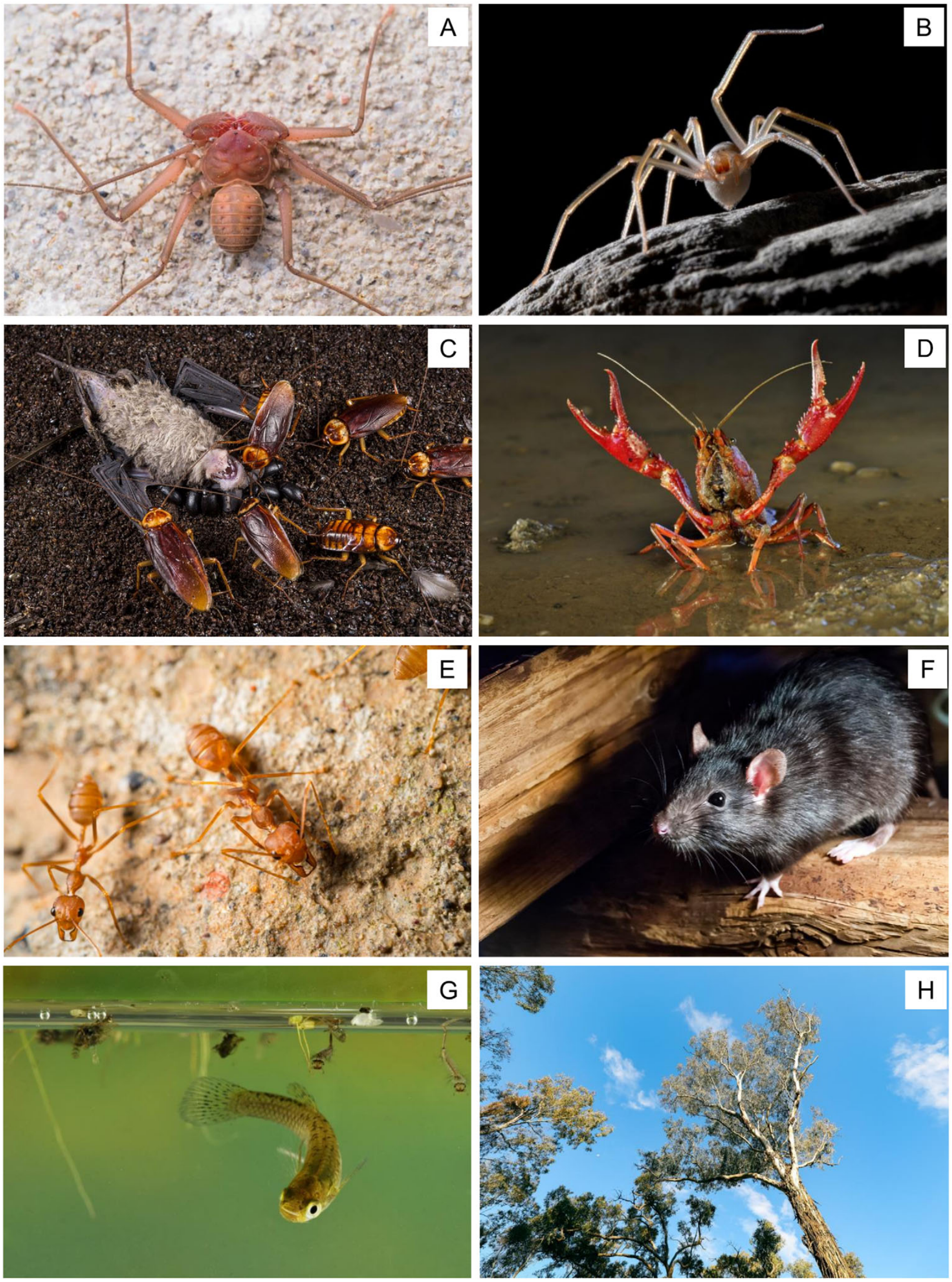

Fig. 2. Examples of alien species invading subterranean habitats. (A) *Charinus ioanniticus* (Kritscher) (Amblypygi); (B) *Loxosceles rufescens* Dufour (Araneae); (C) *Periplaneta americana* (Linnaeus) (Blattodea); (D) *Procambarus clarkii* Girard (Decapoda); (E) *Solenopsis invicta* Buren (Hymenoptera); (F) *Rattus rattus* Linnaeus (Rodentia); (G) *Gambusia* sp. (Cyprinodontiformes); (H) *Eucalyptus tereticornis* Sm. Photograph credits: Enrico Simeon (A), Francesco Tomasinelli (B, D, G), Emanuele Biggi (C), shutterdemon – stock.adobe.com (E), Carlos Aranguiz – stock.adobe.com (F), Caseyjadew – stock.adobe.com (H).

plant's pot; once the pot is placed on the ground in a garden or greenhouse, alien species may find suitable microclimatic conditions (e.g. high moisture) to thrive (Sánchez-García, 2014). Once established, they can disperse and potentially find suitable conditions in subterranean environments. This was seemingly the case for the detritivore *Oxidus gracilis* (CL Koch), known as the 'greenhouse millipede' (Iniesta *et al*., 2020), and the predator *Caenoplana coerulea* Moseley (Suárez, Martín & Naranjo, 2018), recorded in subterranean habitats globally and in the Canary Islands, respectively. The European spider *Kryptonesticus eremita* (Simon) plausibly might have colonised New Zealand *via*

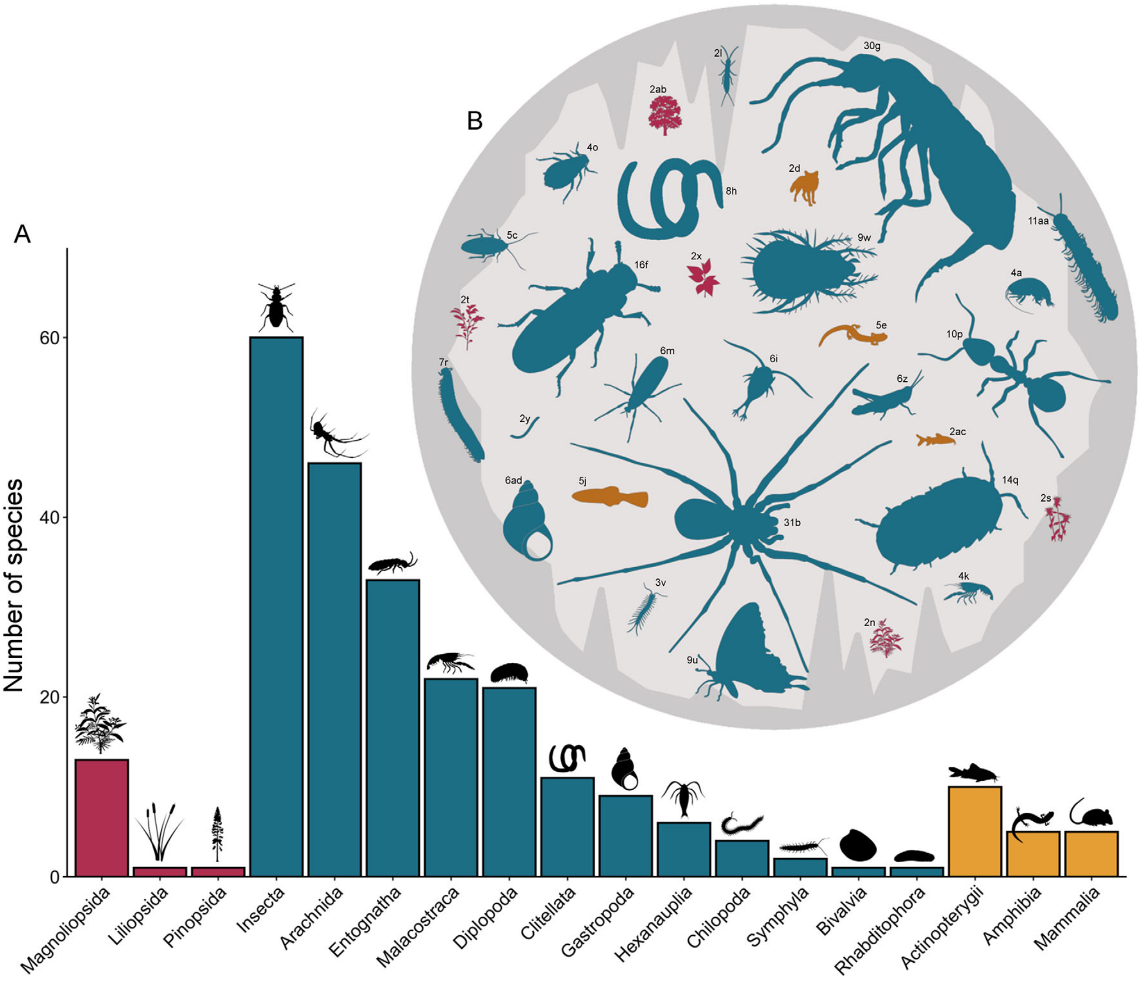

Fig. 3. (A) Barplot representing the number of alien subterranean species within each taxonomic class (total number of species: $N = 246$). (B) Number of alien subterranean species within each taxonomic order (orders with less than two species are not included). The silhouette size for each order is proportional to the number of species detected through the literature survey. Each order is represented by an illustrative example. a: Amphipoda; b: Araneae; c: Blattodea; d: Carnivora; e: Caudata; f: Coleoptera; g: Collembola; h: Crassiclitellata; i: Cyclopoida; j: Cyprinodontiformes; k: Decapoda; l: Diplura; m: Diptera; n: Fabales; o: Hemiptera; p: Hymenoptera; q: Isopoda; r: Julida; s: Lamiales; t: Laurales; u: Lepidoptera; v: Lithobiomorpha; w: Mesostigmata; x: Myrtales; y: Opisthopora; z: Orthoptera; aa: Polydesmida; ab: Sapindales; ac: Siluriformes; ad: Stylommatophora.

shipping containers, considering the proximity between the site of detection of this species and the port of Auckland (Vink & Dupérré, 2011).

The deliberate introduction of alien species represents the third most common pathway (21/64 cases; 32.8%), especially for vertebrates (Fig. 5A), and mostly among omnivores (Table S1). Animals may be deliberately released for their food value, especially in freshwater ecosystems (e.g. Hobbs, Jass & Huner, 1989). For example, the red swamp crayfish *Procambarus clarkii* (Girard) has spread widely throughout freshwater bodies across Europe since its first introduction in Spain (Habsburgo-Lorena, 1978; Souty-Grosset *et al.*, 2016) now representing one of the 100 worst invasive species (DAISIE, 2008). It is increasingly being documented also in aquifers and caves (Mazza *et al.*, 2014; Souty-Grosset *et al.*, 2016; Di Russo *et al.*, 2017; Cilenti *et al.*, 2017).

On rare occasions, alien species have been introduced into subterranean environments for scientific purposes. The olm *Proteus anguinus* Laurenti, a specialised subterranean salamander inhabiting caves in the Dinarides, was deliberately released during the 1940s into a suitable cave in the Mendip Hills, UK (Chapman, 1993). However, there is no evidence that it became established (Lewarne & Allain, 2020). Likewise, *Hydromantes* salamanders have been intentionally released outside their natural range as part of scientific experiments. Evidence suggests the possible establishment of a persistent population capable of reproducing in their new subterranean habitat in the French Pyrenees (Lunghi *et al.*, 2018). Among invertebrates, the beetle *Speonomus normandi hydrophilus* (Jeannel), originally distributed in the French Pyrenees, has been experimentally introduced into Dzwonnica Cave (Poland). Interestingly, there is evidence for molecular divergence between the native and introduced

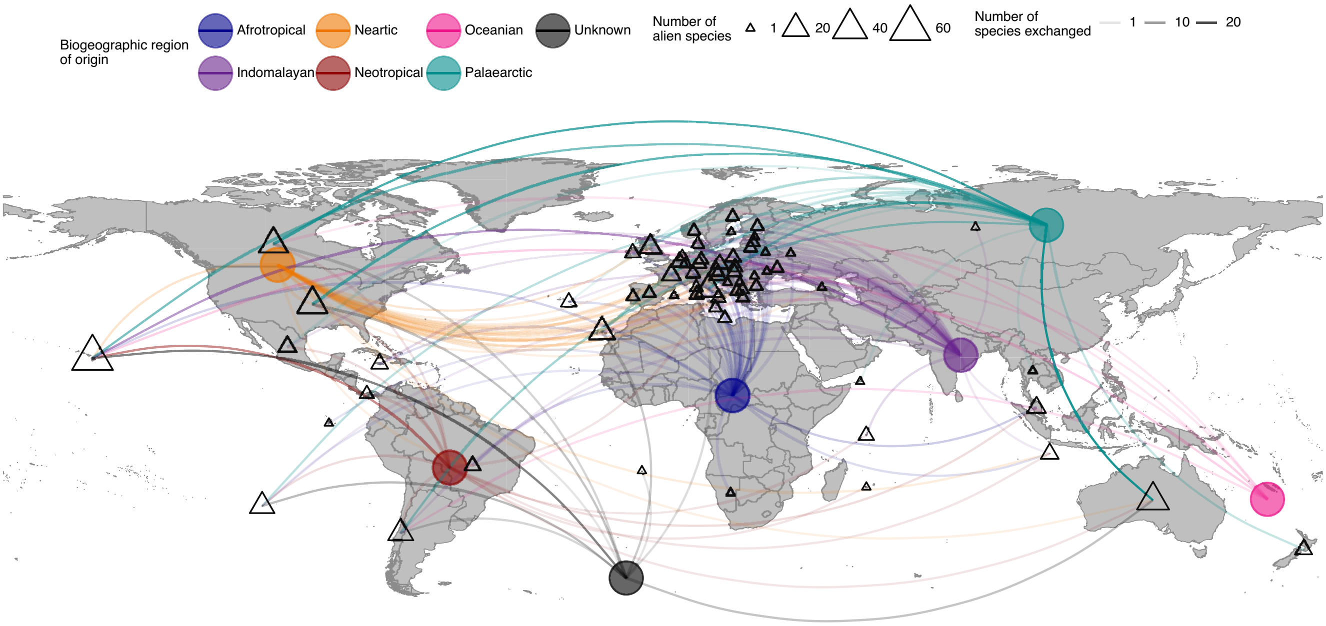

Fig. 4. Exchanges of subterranean alien taxa among biogeographic regions and countries. Circles represent one of the six bioregions (the black circle marks species with a global and/or uncertain origin; its position is arbitrary). Triangles represent the number of alien species detected in each country, with the size of the triangles proportional to the number of species. Lines represent the number of alien species exchanged between bioregions and countries, with the thickness of the lines proportional to the number of species.

populations, suggesting that the local conditions might have an important influence on haplotype diversity of both populations (Kocot-Zalewska, Domagała & Lis, 2021).

Finally, escapes represent the least frequent form of introduction (6/64 cases; 9.4%), although they are more common for vertebrates (Fig. 5A). Escape is considered among the most common pathways for alien plants and vertebrates (Saul *et al.*, 2017), especially through the horticulture trade (Turbelin *et al.*, 2017). This route was mostly represented among omnivores in our database (Table S1).

(3) What are the impacts of alien species in subterranean habitats?

Our results reveal that in most cases the impact of alien species in subterranean ecosystems is unknown (280 out of 362 cases; 77.3%), whereas they have negative biological consequences in 82 out of 362 cases (22.7%).

The outcome was specified in our database in only 76 cases. Of these, 65 out of 76 cases (85.5%) have negative repercussions at the species/population level and 49 out of 76 (41.3%) on ecosystems/habitat.

Information on the mechanisms through which alien species impact native subterranean organisms and/or ecosystems was available for 67 cases, with the most important being competition (40/67 cases; 59.7%) and predation (26; 38.8%), followed by disease transmission (7; 10.4%) and parasitism (4; 6.0%). There were single records of negative impacts *via* grazing/herbivory/browsing, poisoning/toxicity, rooting/digging, interaction with other invasive species, and hybridisation. Information about mechanisms was lacking for the majority of cases included in our database (295 out of 362 cases; 81.5%) (Fig. 5B).

Competition of alien species with native organisms was most prevalent for plants and vertebrates (Fig. 5B), and mostly affects omnivores and primary producers (Table S1). Many alien species have traits that allow them to outcompete residents once they establish themselves in new areas. This is true for *P. clarkii* which occur at greater densities and tend to be more active in comparison with indigenous crayfish species (Reynolds, 2011). The presence of *P. clarkii* in subterranean ecosystems is widely reported (e.g. Mazza *et al.*, 2014; Souty-Grosset *et al.*, 2016; Di Russo *et al.*, 2017; Cilenti *et al.*, 2017), and established populations are able to thrive over a wide range of biotic and abiotic conditions from tropical to temperate zones (Gherardi & Panov, 2009; Siesa *et al.*, 2011). Likewise, the non-subterranean spider *N. mogera* (Yaginuma) appears to be outcompeting local populations of the spider *Erigone stygia* Gertsch in the mid- to high-elevation caves on Hawai'i Island. Due to the constant supply of new individuals from surface habitats, the alien spider is replacing *E. stygia*, and probably exploits same prey (Howarth, 1978).

Although plants cannot colonise light-deprived underground environments, roots may penetrate the ceilings of shallow caves and other superficial subterranean habitats competing with local species and causing management issues (Howarth *et al.*, 2007).

Predation represents the second most common impact mechanism in subterranean ecosystems (38.8% of cases), mostly among invertebrates and vertebrates (Fig. 5B), and especially for omnivores (Table S1). The red fire ant

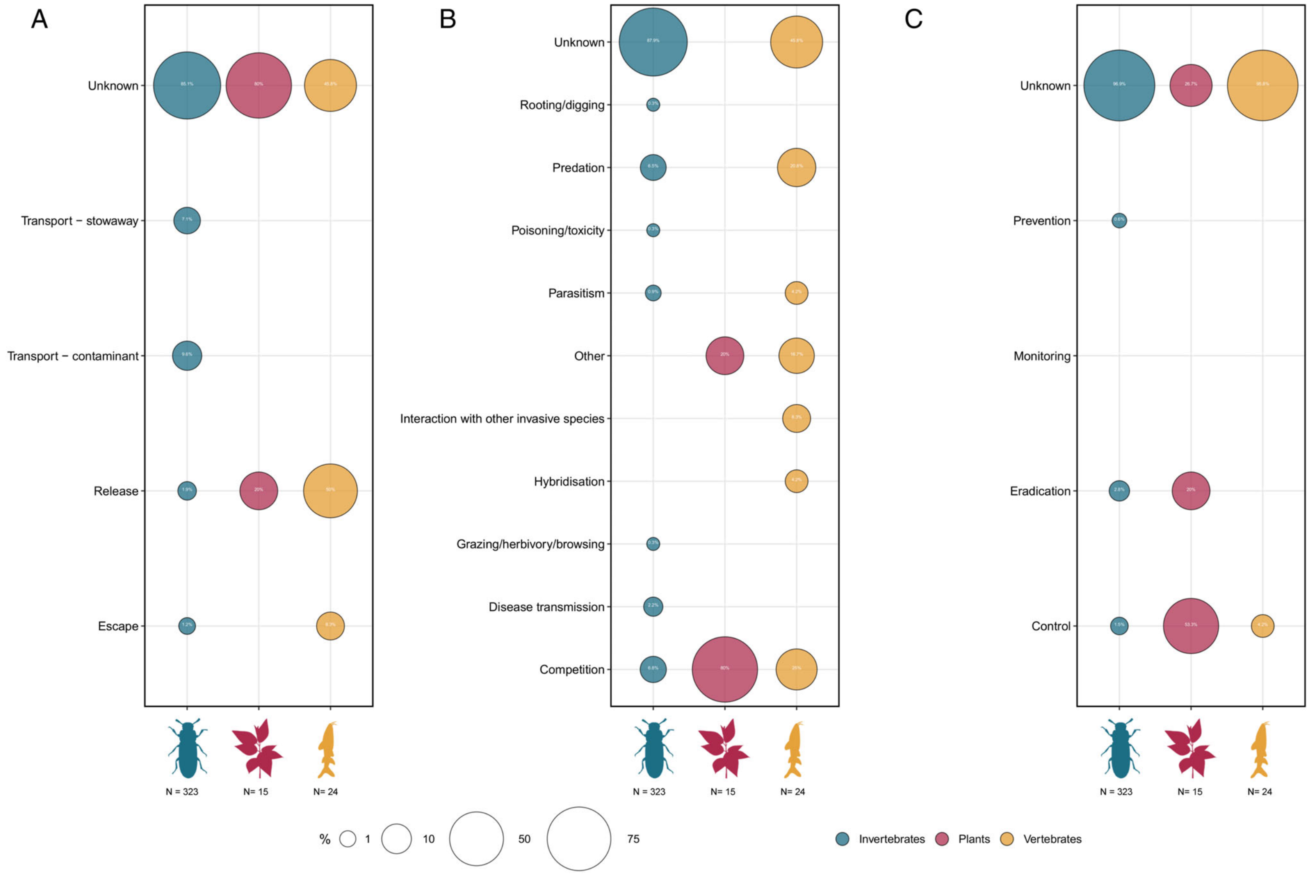

Fig. 5. (A) Introduction pathways for the 246 subterranean alien species into the recipient area. (B) Mechanisms of impacts in the 362 cases studied present in our database (mechanisms with a frequency equal to zero are not included). (C) Management activities discussed in the 362 cases studied present in our database. Circle size is proportional to the percentage of species or cases for each group (invertebrates, vertebrates, and plants). *N* indicates the total number of cases for each group.

S. invicta Buren represents one of the most harmful predators in subterranean ecosystems (Elliott, 1992, 2000; Taylor, Krejca & Denight, 2005; Cokendolpher *et al.*, 2009; Pape, 2016). *S. invicta* is considered one of the 14 worst invasive alien insect species worldwide (Lowe *et al.*, 2000) and is included within the top 100 of the World's worst invasive species by the IUCN (Boudjelas *et al.*, 2000). Although it is not strictly subterranean, it often constructs mounds near cave entrances because of suitable microclimatic conditions (Elliott, 1993). From there, individuals enter the caves and prey efficiently on numerous subterranean species, including several endangered species (Elliott, 1993; Cokendolpher *et al.*, 2009).

Some vertebrates can be efficient predators in subterranean ecosystems and may pose a serious threat to cave-dwelling species. The presence of rats (*Rattus rattus*), a cosmopolitan pest widely recognised as one of the most damaging invasive species worldwide [Global Invasive Species Database (GISD), 2020], has been highlighted in numerous caves in the Hawaiian Islands. Rats enter caves in search of water and food and may prey on native species (Howarth & Stone, 2020).

Although underrepresented in the literature, subterranean alien species can also transmit disease (10.4%) or have impacts *via* parasitism (6.0%) (Fig. 5B). Introduction of the guppy *Poecilia reticulata* Peters into the subterranean karst habitat of Christmas Island (Australia) is considered a threat due to both its highly predatory activity and to its potential transmission of a parasite (Asian fish tapeworm *Bothriocephalus acheilognathi* Yamaguti) which could threaten eleotrid fish populations (Humphreys, 2014). The bed bug *Cimex lectularius* Linnaeus has been recorded to feed on bats and probably transmits *Trypanosoma cruzi* Chagas (Reeves, 1999). The browndog tick *Rhipicephalus sanguineus* Latreille introduced into North America from Europe is a vector for several diseases (Reeves, 1999).

(4) What are the socio-economic impacts of alien species in subterranean ecosystems?

Of all alien species found in subterranean ecosystems, only 2.2% have been associated with a socio-economic impact, although these costs have not been quantified in detail. Information on costs associated with alien species in subterranean ecosystems is very limited. This may not be surprising as many of these species are invertebrates, which are generally underrepresented in the literature (Cardoso *et al.*, 2011; Titley, Snaddon & Turner, 2017). Additionally, when a species has no or little impact in a certain habitat, there will be no assessment of damage or intervention costs.

A recently developed database on the economic costs of invasive alien species globally (Diagne *et al.*, 2020*a*), and associated studies using this database, provide an opportunity to look in more detail at the economic costs associated with alien species present also in caves.

Among these, only *S. invicta* is known to be associated with substantial costs (Angulo *et al.*, 2022). This species is among the most notorious invasive species in subterranean ecosystems, and is considered a serious land invertebrate pest. Its invasive behaviour leads to impacts on human health, livestock, biodiversity, crops, and machinery (Wojcik *et al.*, 2001). Elliott (1993) evaluated the efficacy and relative cost of different treatment methods in subterranean habitats, but a general estimate of the socio-economic cost of this species in such habitats is still lacking.

Some species are associated with very high economic costs in other habitats. For example, of the 100 World's worst invasive alien species, *R. rattus* has the second highest associated costs (Cuthbert *et al.*, 2021), however, these reported costs resulted mainly from severe impacts on resident animal populations on islands (e.g. through predation of birds' eggs) and from efforts to eradicate them (e.g. Genovesi, 2005; Parkes, Byrom & Edge, 2017). The economic costs associated with this species in subterranean ecosystems remain largely unknown (Howarth & Stone, 2020).

Although unquantified, the introduction of alien species into subterranean ecosystems may also have social costs. These can include a decrease or loss of heritage value of cave-dwelling native species (Souty-Grosset *et al.*, 2016). For example, the presence of alien crayfish can lead to the disappearance of festivals celebrating native crayfish (Reynolds & Souty-Grosset, 2011).

(5) What are the management interventions used to protect subterranean habitats?

Management interventions have been used in only in a limited number of cases (22/362) in subterranean ecosystems (Fig. 5C). Furthermore, the effectiveness of these interventions has seldom been tested statistically (Mammola *et al.*, 2022), and most knowledge on eradication activities remains qualitative (Simberloff, 2002; Genovesi, 2005). Eradication actions have been undertaken to counteract the spread of the fire ant *S. invicta* in the southern USA. The most efficient methodology seems to be the use of boiling water to kill ants in the nest. Even though this is labour-intensive, it avoids the problem of non-target species consuming insecticidal baits (Elliott, 2000). However, it is not a cost-effective method over large areas (Elliott, 1992, 1993). The trapping and hand removal of *P. clarkii* from subterranean habitats has reduced populations of this species, but has not led to its eradication from these ecosystems (Mouser *et al.*, 2018).

When eradication fails, long-term control activities can limit the impact of an alien species, reducing its density and abundance (Mooney *et al.*, 2005). Several methods to control the dispersal of *P. clarkii* have been tested, with a synergistic approach using different methodologies often the most successful (Souty-Grosset *et al.*, 2016). For rats, control activities are generally carried out by both public and private agencies, but caves usually are not included in such efforts (Howarth & Stone, 2020).

Prevention actions can stop a species from colonising new areas (Mooney *et al.*, 2005). For example, the installation of artificial barriers can be a useful mechanism to prevent the entrance and spread of alien species in subterranean streams (Mouser *et al.*, 2018). However, besides the cost implications of such barriers often being high, they may alter the flow regime and/or microclimatic conditions, while also preventing the movement of organisms in stream ecosystems (Ellis & Jones, 2013).

(6) Are there common traits shared by alien species that successfully establish in subterranean ecosystems?

Of the 246 alien species listed in our data set, 127 (51.6%) are considered to be successfully naturalised in subterranean habitats. Insects and arachnids make up the greatest proportion of naturalised species, with other invertebrate groups (gastropods and myriapods) underrepresented. Approximately one third of the species recorded in subterranean habitats are not considered to be established (73/246 species). No information on establishment success was available for 46 species (18.7%).

Only some of these alien species exhibit adaptations to subterranean life (90/246; 36.6%), including depigmentation, eye loss/reduction, or a preference for dark and humid habitats. This limited number of alien species strictly adapted to subterranean environments (e.g. *Proteus anguinus*, *Parabathyscia dematteisi*) mostly pertains to escapes of species

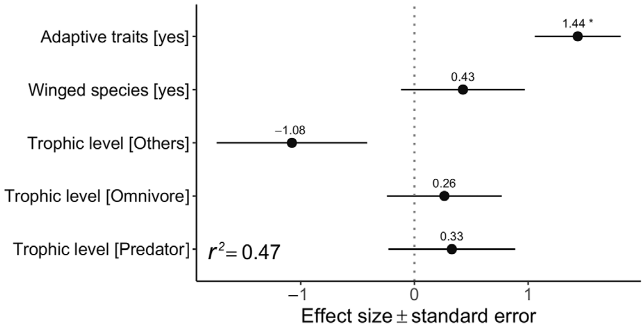

Fig. 6. Effect sizes for the Bernoulli generalised linear mixed model assessing the relationship between species traits and the probability of becoming established in subterranean ecosystems. See Table 2 for model results. *, $P < 0.001$.

Table 2. Estimated regression parameters according to a Bernoulli generalised linear mixed model (GLMM) investigating the drivers of established alien species in subterranean habitats.

Predictor	Estimate	S.E.	z	P
Intercept	−0.693	0.480	−1.445	0.148
Adaptive trait [Yes]	1.436	0.378	3.803	<0.001
Presence of wings [Yes]	0.427	0.544	0.784	0.432
Trophic level [Others]	−1.078	−0.662	−1.628	0.104
Trophic level [Omnivore]	0.263	0.505	0.522	0.602
Trophic level [Predator]	0.329	0.558	0.590	0.556

For predictor variables, we report in square brackets the level that is being tested. For the variables 'Adaptive trait' and 'Presence of wings', the baseline level used in the analysis is 'No'. For the variable 'Trophic level', the baseline level is 'Detritivores'.

introduced into subterranean habitats for scientific purposes (e.g. Chapman, 1993; Lewarne & Allain, 2020). This is probably due to the high sensitivity of such species to even small environmental variations (e.g. Barr & Kuehne, 1971; Howarth, 1980; Culver, 2005; Nicolosi *et al.*, 2021) limiting their dispersal outside a subterranean environment.

Our modelling showed that the presence of adaptive traits is the strongest predictor of the probability that a species will become established in a subterranean habitat (binomial GLMM: estimated β ± SE: 1.44 ± 0.38, $z = 3.80$, $P < 0.001$; Fig. 6; Table 2). Additionally, the probability of establishing in a subterranean habitat was lower for species in the trophic level 'Others' (including herbivores, primary producers and parasites in this analysis) compared to detritivores, although this did not reach statistical significance (β ± SE: −1.08 ± 0.66, $z = -1.63$, $P = 0.10$). No other traits were found to exert a significant effect on the probability of becoming established in a subterranean habitat (Table 2). The regression model explained 47% of the variance (conditional r^2: 0.47), of which over 28% was attributable to species taxonomy.

IV. CONCLUSIONS

(1) Due to their simplified trophic web, low species diversity, and high spatial confinement, subterranean ecosystems are generally considered more vulnerable than surface ecosystems to anthropogenic disruption (Mammola *et al.*, 2019). Whilst many authors have suggested that the presence of alien species may contribute significantly to the decline of subterranean species and ecosystems (e.g. Mazza *et al.*, 2014; Suárez *et al.*, 2018; Howarth & Stone, 2020), the true extent of their impact remains unclear (Mammola *et al.*, 2020). Furthermore, our understanding is geographically and taxonomically biased. In-depth studies remain needed to understand the significance of alien species in subterranean ecosystems and how they affect the subterranean biota. This review provides the first comprehensive global synthesis of alien species in subterranean ecosystems. By organising the available information, it is hoped that this study will stimulate work to fill major knowledge gaps.

(2) From the available literature, the number of alien species observed in subterranean habitats is rather small. This is in stark contrast to surface systems, where databases on alien species are available at continental, regional, or national scales resulting from large international collaborations such as the Global Invasive Species Database (http://www.issg.org/database), the Global Register of Introduced and Invasive Alien Species (www.griis.org; Pagad *et al.*, 2018), and alien species inventories for Europe (Roy *et al.*, 2020). Interestingly, none of these databases report specific information on alien species in subterranean ecosystems, with caves and related environments generally not even included as a separate habitat.

(3) Although only limited data are available, it appears that only a few alien species represent a threat to subterranean ecosystems and to the species living therein. To colonise subterranean systems, alien species need to overcome the strong ecological filter imposed by the absence of light and the scarcity of food (Culver & Pipan, 2019). Successful invaders must therefore possess traits that enable them to cope with these environmental constraints (Reeves, 1999; Mammola, 2017). This was confirmed by our analysis, which suggested that the main predictor explaining the probability of a species becoming established in subterranean systems is the presence of pre-adaptive traits.

(4) Interactions between human activities and climate change might accelerate the spread of alien species into new environments, including subterranean habitats. However, investigations on the links between invasions and environmental changes in subterranean habitats are still rare (but see Mammola & Isaia, 2017). A common framework for the study of the consequences of climate changes and the routes of transport, establishment and impacts of alien species will be necessary to understand long-term consequences for subterranean ecosystems.

(5) Researchers in the field of subterranean biology should report the presence of alien species when preparing species inventories in addition to recording the presence of endemicity and rarity. Greater awareness of the presence and distribution of alien species will allow a greater understanding of the potential distribution and spread of alien invertebrate species in subterranean habitats, laying the foundations for future management practices and interventions. It is

currently difficult to recommend management practices in the absence of well-documented relationships between native and alien species (Reeves, 1999). Adequate and rapid dissemination of information on alien species will be crucial to prevent and manage their expansion effectively (CBD, 2000), because impacts can occur in different environments through a variety of mechanisms (Ricciardi *et al*., 2013). We need to work towards the efficient prevention, early detection, rapid response, and management of biological invasions in these fragile habitats.

V. ACKNOWLEDGEMENTS

This review was prepared within the framework of the PRIN SHOWCAVE '*A multidisciplinary research project to study, classify and mitigate the environmental impact in tourist caves*' – code 2017HTXT2R (PI: Marco Isaia), funded by the Italian Ministry of Education, University and Research. S. M. acknowledges support from the European Commission *via* the Marie Sklodowska-Curie Individual Fellowships project number 882221 ('*Testing macroecological theory using simplified systems*') and the Biodiversa+ project DarCo ('*The vertical dimension of conservation: A cost-effective plan to incorporate subterranean ecosystems in post-2020 biodiversity and climate change agendas*'). Open Access Funding provided by Universita degli Studi di Torino within the CRUI-CARE Agreement.

VI. AUTHOR CONTRIBUTIONS

G. N. conceived the idea, with suggestions by M. I. and S. M. G. N. collected data. G. N. and S. M. analysed the data and prepared the figs G. N. and S. M. led the writing. M. I. revised the text and provided additions to the final draft. L. V. provided arguments on alien species and estimations of costs.

VII. DATA AVAILABILITY STATEMENT

The literature database and R code to reproduce the analyses supporting this study are available in Figshare (https://doi.org/10.6084/m9.figshare.21779045.v2).

VIII. REFERENCES

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IX. SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. PRISMA flow diagram (*sensu* Moher *et al.*, 2009) depicting the flow of information through the different phases of the systematic literature search.

Fig. S2. The number of articles published per year on subterranean alien species in subterranean habitats.

Table S1. Full list of papers extracted in our systematic survey and respective extracted metadata.

(*Received 8 July 2022; revised 5 January 2023; accepted 10 January 2023*)