HUMAN-INDUCED ALTERATIONS OF THE MYCOBIOTA IN AN ALPINE SHOW CAVE (ITALY, SW-ALPS)

SPREMEMBE MIKOBIOTE, KI JIH V TURISTIČNI ALPSKI JAMI (ITALIJA, SZ ALPE) POVZROČI ČLOVEK

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Abstract   UDC 551.44:582.28(450.215)
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Stefano Mammola, Simone Di Piazza, Mirca Zotti, Giovanni Badino, Marco Isaia: Human-induced alterations of the mycobiota in an Alpine show cave (Italy, SW-Alps)
Anthropogenic alterations in show caves are well documented in scientific literature. One of the main sources of perturbation derives from visitors, acting as carriers of propagules and organic matter in the cave ecosystems. Such perturbation generally results in a significant alteration of the natural subterranean microbiota. In this study, we characterize the mycobiota of the superficial alluvial sediment (0–5 cm) of the Bossea show cave (NW-Italy) over two sampling seasons, aiming to investigate whether anthropogenic rather than natural factors contribute to the colonization and proliferation of alien mycobiota in the cave environment. We placed eighteen sampling plots at different distances from the touristic pathway that crosses the cave. The distance from the touristic pathway was used as proxy to sort the sampling plots in three groups according the degree of anthropogenic disturbance (high: 0–2 m; intermediate 2–40 m; low: >100 m). Moreover, in order to elucidate the potential effect of other factors, we introduced in our analysis the distance from the subterranean river and the distance from the cave entrance. In each plot, we collected two samples of alluvial sediment, in winter and summer. Fungi were isolated from each sample and identified by means of an integrated morphological approach. We observed a rich and diversified fungal community – 63 taxa of Zygomycota and Ascomycota plus several unidentified yeasts – consisting of both native and alien species. Regression analysis points out a decrease of the abundance and the diversity of viable propagules at increasing distances from the touristic pathway rather than the cave entrance or the

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Stefano Mammola, Simone Di Piazza, Mirca Zotti, Giovanni Badino, Marco Isaia: Spremembe mikobiote, ki jih v turistični alpski jami (Italija, SZ Alpe) povzroči človek
Antropogene spremembe v turističnih jamah so v znanstveni literaturi dobro opisane. Glavni vir motenj so obiskovalci, ki za jamske ekosisteme delujejo kot vektorji živih propagul in organskih snovi. Te motnje običajno povzročijo občutno spremembo naravne mikrobiote v podzemlju. Na podlagi dveh vzorčevanj smo v študiji podrobno opisali mikobioto na površini aluvialnih sedimentov (0–5 cm) v turistični jami Bossea (SZ Italija). Namen raziskave je bilo ugotoviti, ali h kolonizaciji in proliferaciji nejamske mikobiote v jamskem okolju bolj prispevajo antropogeni kot naravni dejavniki. V jami smo na različnih razdaljah od turistične poti določili osemnajst mest vzorčenja. Razdaljo od turistične poti smo uporabili za razvrstitev vzorčnih mest v tri skupine glede na stopnjo človekove motnje (visoka: 0–2 m; vmesna 2–40 m; nizka: > 100 m). Da bi opredelili morebitne vplive drugih dejavnikov, smo v naši analizi dodatno upoštevali še razdaljo od podzemne reke in od vhoda v jamo. Pozimi in poleti smo na vsaki točki odvzeli dva vzorca aluvialnega sedimenta. Iz vsakega vzorca smo izolirali glive ter jih identificirali glede na njihove morfološke značilnosti. Ugotovili smo bogato in raznoliko združbo gliv – 63 taksonov Zygomycota in Ascomycota in še nekaj neidentificiranih kvasovk, značilnih jamskih in zunanjih vrst. Regresijska analiza je pokazala, da se zmanjšuje abundanca in diverziteta viabilnih propagul z oddaljevanjem od turistične poti bolj kot z oddaljevanjem od jamskega vhoda oziroma podzemeljskega vodotoka. V skladu s to ugotovitvijo se zdi, da turisti pasivno prenašajo zunanji organski material, kar lahko razloži večjo razširjenost

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subterranean river. Accordingly, the exogenous organic materials passively conveyed by tourists, possibly explains the higher proliferation of alien species in the proximity of the touristic pathway. There was also a seasonal variation in the diversity and abundance of propagules, which we interpreted in light of the peculiar microclimate of the cave. In a second step, we used Indicator Species Analysis to identify the most representative species of the different levels of anthropogenic disturbance. In particular, *Aspergillus spelunceus* was found to be indicator of low disturbance, whereas *Mucor corticola* and *A. asperescens* were found to be indicators of intermediate and high disturbance, respectively.
Key words: anthropic disturbance, alluvial sediment, alien species, airborne fungi, environmental impact.

nejamskih vrst v bližini turistične poti. Ugotovili smo tudi sezonsko variabilnost v diverziteti in abundanci propagul, kar lahko razložimo s posebnimi mikroklimatskimi razmerami, ki vladajo v jami. V naslednjem koraku smo za identifikacijo reprezentativnih vrst, ki kažejo različne nivoje antropogenih motenj, uporabili indikatorsko vrstno analizo. Gliva *Aspergillus spelunceus* se je izkazala kot indikatorska vrsta za nizko stopnjo motnje, *Mucor corticola* je bila indikator srednje, *A. asperescens* pa indikator visoke stopnje motnje.
Ključne besede: antropogene motnje, splošni linearni mešani modeli, glive v zraku, vpliv na okolje.

INTRODUCTION

Over the past few decades, interest for the underground karst environments and their natural wonders has grown remarkably, not only from the speleological or scientific point of view, but also from an economic perspective. The so-called "show caves" are caves made accessible to the general public for touristic purposes, managed by a government or commercial organization. Unlike wild caves, paying visitors experience the cave via constructed trails, guided tours, artificial lighting and regular opening hours (Wilson 2005). Since the first evidence of cave visitors in 1213 in the Postojna Cave and the early experiments with electric light in Australian caves in 1881, cave tourism grew considerably (Cigna 2005). The impressive numbers of visitors – up to 500,000 visitors/year/cave – and the profits deriving from such activities have recently acquired substantial importance at global scale (International Show Cave Association 2015).

Caves are delicate and sheltered ecosystems, because of their peculiar habitat conditions, spatial confinement, climatic stability and the conservation value of their biodiversity (Culver & Pipan 2009). The susceptibility of caves to disturbance, especially in show caves, is well documented (e.g., Fernandez-Cortes *et al.* 2011). It is understood that the impressive tourist flow and the associated transformations contribute significantly to alter cave equilibrium, as a massive amount of additional energy is introduced in the system. Energy release mostly derives from visitors – primarily in form of heat and CO_2 – from artificial lights and other indirect sources, resulting in profound climatic and energetic perturbations of the entire cave ecosystem (Cigna 1993). On top of that, human visitors carry propagules and organic matter in caves (Krajick 2001), influencing the availability of food resources with straightaway fallouts on the subterranean trophic webs (Chelius *et al.* 2009). The joint effect of

visitors and artificial lights often results in the colonization and proliferation of alien organisms, such as the so-called "lampenflora" (algae, diatoms, ferns, mosses), bacteria and fungi (Vanderwolf *et al.* 2013; Falasco *et al.* 2014).

These alien organisms induce dramatic ecological changes, both from the biotic and the abiotic point of view. Changes in species interactions, perturbations of nutrient cycles and structural damages to speleothems are among the most cited threats associated with caves opened to public (Sánchez-Moral *et al.* 1999, 2005; Fernandez-Cortes *et al.* 2011; Piano *et al.* 2015).

Among microorganisms, fungi are exceptionally diverse, with about 98,000 described *taxa* (Kirk *et al.* 2008). In general, the great sporulation ability and anemophily of fungi allows them to survive in a wide variety of environments (Magan 2007), including hypogean ones. With 1,029 species documented from caves (Vanderwolf *et al.* 2013), fungi and yeasts represent an important component of cave trophic webs (Nováková 2009), where they play a key role in recycling organic matter and making available nutrients for other organisms. In recent years, there was an increasing interest about mycobiological researches in cave environments (Rampelotto 2010), generally aimed at looking for new species (Selbmann *et al.* 2005), assessing diversity (Gostinčar *et al.* 2010; Selbmann *et al.* 2012), and isolating microorganisms exploitable for biotechnological purposes (Gadd 2007). An increasing number of studies have also investigated whether humans visitors has an impact on the fungal diversity – reviewed in Vanderwolf *et al.* (2013).

Given their peculiar role in nutrient cycling, the perturbation of the cave mycobiota can result in profound alteration of the cave ecosystem. Several studies have shown that different groups of fungi are involved

in biogeochemical process within caves (Gadd 2007) and how alien species damage caves – e.g., Lescaux Cave in France (Bastian *et al.* 2010) and the Castañar de Ibor Cave in Spain (Jurado *et al.* 2010).

We characterize quantitatively and qualitatively the mycobiota of the superficial alluvial sediment (*sensu* Sasowsky, 2007) of the Bossea show-cave (NW Italy). The aim of the study is to analyze whether visitors, in contrast to natural sources such as natural air currents and running waters, contribute to the colonization and the proliferation of the alien mycobiota. In particular, we hypothesize that the proximity to the touristic pathway – that we interpreted as a *proxy* for anthropogenic disturbance – affects positively both the diversity and the abundance of the mycobiota.

MATERIAL AND METHODS

STUDY AREA

The Bossea show cave (speleological cadastre: 108 Pi/CN) is part of a karst system developed in a sub-basin of the Corsaglia watershed, located in the SW-Italian Alps – mount Merdenzone, municipality of Frabosa Soprana, Province of Cuneo, NW-Italy. The Bossea cave is included in Site of Community Importance (SCI) IT1160026, and is set in a narrow belt of limestone and dolomitic limestone imposed upon permo-carboniferous rocks – quarzites and porfiroids (Civita *et al.*, 2005).

The cave is particularly rich in water since it is crossed by a subterranean river flowing inside the cave with a flow rate ranging from 50 to 1200 l/sec. The cave has a total ground plan development of 2,800 m and an ascending structure. It opens at 836 m a.s.l. and reaches 1,040 m at its highest point. It was firstly explored in 1850 and opened to public in 1874. With 150 years of tourist frequentation, the Bossea cave is the oldest Italian cave open to public. The current touristic flow rate is estimated to be 16,000 visitors/year (Bossea management office 2015).

A gate with open bars – i.e. allowing natural airflow – at the cave entrance restricts public access (Fig. 1a). Visitors have access to the touristic section of the cave via a narrow corridor of nearly 100 m length, which is often used for contemporary art expositions. After the corridor and along the touristic pathway (Fig. 1b), visitors have access to a consecutive series of vast rooms – i.e., "Sala Garelli" of 100 x 60 x 40 m. The touristic pathway ends at the Ernestina Lake (Fig. 1c) at 949 m a.s.l., nearly 1 km away and 113 m higher than the entrance (Fig. 2). After the Ernestina Lake, the non-touristic section begins. A long active canyon of 3–6 m wide and up to 40 m high follows, ending in a system of submerged syphons, which have been partially explored by speleo-divers (Gregoretti 1991).
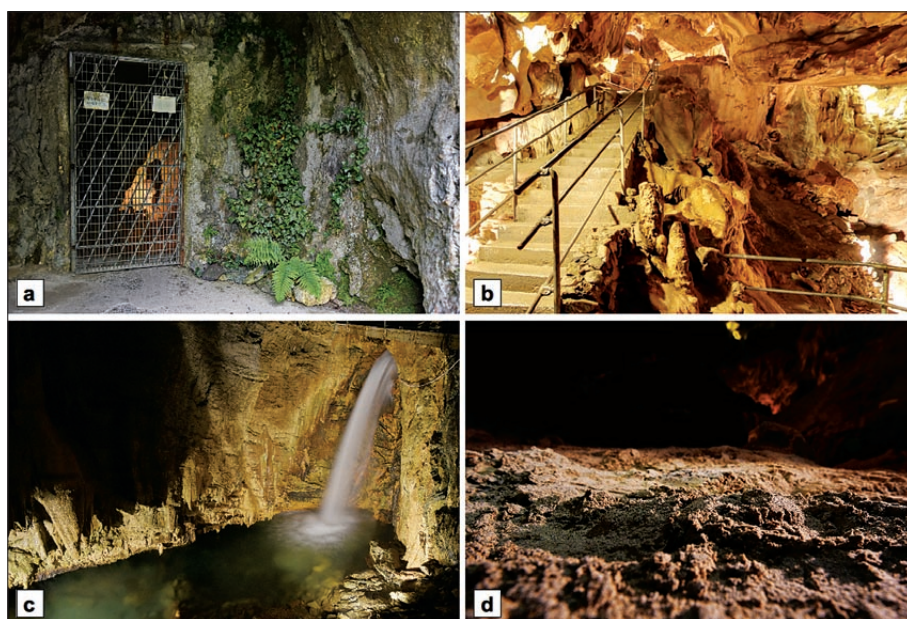

Fig. 1: The Bossea show cave. a) Cave entrance; b) Touristic pathway; c) Ernestina lake and the waterfall; d) Alluvial sediment sampled in this study. Photo credits/By courtesy of: Francesco Tomasinelli.
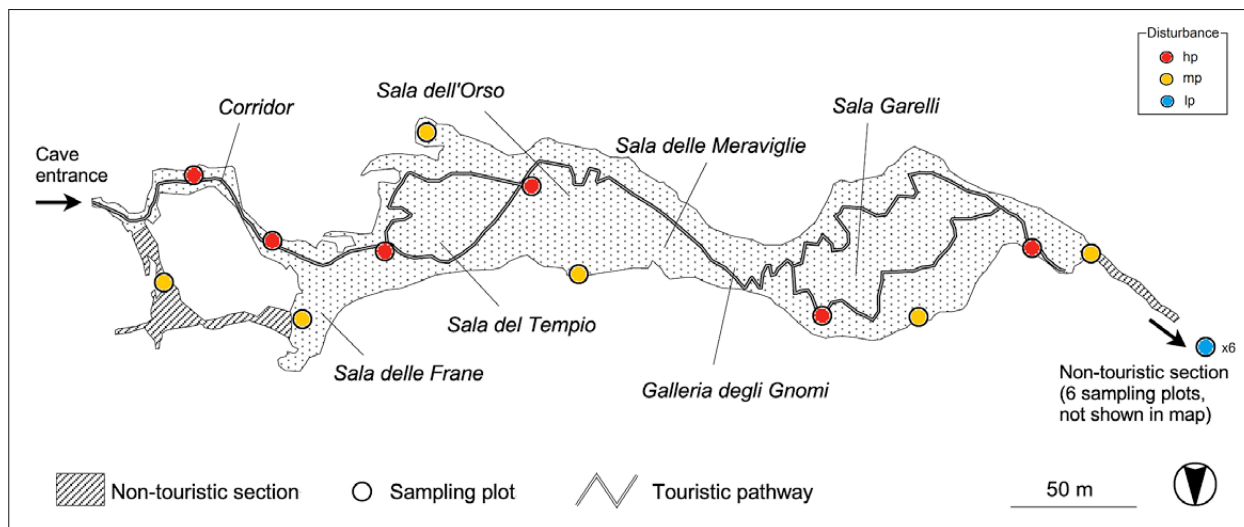
Fig. 2: Ground plan drawing of the touristic section of the Bossea show cave (modified from Capello 1954) with indications of the sampling plots and the assigned level of disturbance. Red points: high disturbance (hp), yellow points: intermediate disturbance (mp), blue points: low disturbance (lp, not shown on the map).

SAMPLING DESIGN

In order to evaluate the effect of touristic impact on the cave mycobiota, we selected 18 squared sampling plots of 0.25 m², placed in different sections of the cave (Fig. 2). For each sampling plot we measured the distance from the cave entrance ('DST_entrance'), the distance from the river ('DST_river'), and the distance from the touristic pathway ('DST_path'). According to the distance from the touristic pathway, we grouped the sampling plots in three categories: high disturbance ('hp'; 0–2 m from the pathway, 6 plots), intermediate disturbance ('mp'; 10–40 m from the pathway, 6 plots) and low disturbance ('lp'; over 100 m from the pathway, 6 plots). Sampling was performed in January and repeated in August 2013 reaching in total 36 samples (6 plots x 3 groups x 2 seasons). During the first sampling in winter, each point was marked in order to relocate it in summer. Alluvial sediment (Fig. 1d) was sampled randomly within the square plot by means of sterile tools. Sediment was collected up to 5 cm depth. The sampling method ensured to collect mainly airborne fungi. Samples were stored in sterile Falcon° tubes (45 ml) and conserved in a thermal bag until the arrival at the laboratory.

Fungi were isolated using the modified dilution plate protocol (Gams *et al.* 1987) on three media (Sigma-Aldrich°): MEA+C (Malt Extract Agar added with Chloramphenicol), RB (Rose Bengal agar), and SAB (Sabouraud agar). We obtained the initial dilution by mixing 1 g of each alluvial sediment sample with 10 ml of sterile water. Then we performed a serial dilution up to 1: 100,000 concentration and finally we inoculated 2 ml of diluted solution on each media in duplicate. We opted for streak 2 ml of solution after preliminary inoculation tests carried out using different portion of sample and dilution. This protocol allowed us to count, identify and isolate vital strains from the environment surveyed. The mycobiota was identified according to cultivation methods by observing their micro- and macro-morphological characters, and also considering their different trophic and physiological requirements (Pitt 1979; Hoog 1978; Domsch *et al.* 2007). Moreover, in order to identify critical species, molecular analyses (ITS and ß-tubulin locus DNA sequence) were performed and compared to BLAST (Basic Local Alignment Search Tool) results. The isolated strains were conserved in the culture collection of Mycological Laboratory of DISTAV (University of Genoa, Italy).

DATA ANALYSIS

REGRESSION ANALYSIS

We analyzed data with Generalized Linear Models (GLMs) in R environment (R Development Core Team 2013). For each plot, we used as dependent variables the concentration of colony-forming unit (CFU/g*10⁶; hereinafter CFU) and the Shannon-Wiener diversity

index (log 10; hereinafter SH) calculated for each plot.

We included the distance of the sampling plot from the touristic pathway ('DST_path'), the distance from the entrance of the cave ('DST_entrance'), the distance from the river ('DST_river'), and the sampling season (categorical variable 'SEA' made up of two levels: Winter and Summer) as covariates in the analysis. We interpreted the distance from the touristic pathway as *proxy* for the disturbance caused by tourists visiting the cave, i.e. the higher the distance of the sampling plot from the path, the lesser the disturbance. The distance from the cave entrance was interpreted as *proxy* for the evaluation of the effect of anemochoric transportation (i.e. air currents) and the distance from the river as *proxy* for hydrochoric transportation (i.e. running water) of spores and propagules. The seasonal categorical variable was introduced in the regression models to take into account possible seasonal effects.

We carried out data exploration following Zuur *et al.* (2010) protocol. According to Zuur *et al.* (2009, 2010) the inclusion of outliers and highly correlated predictors in the regression analysis leads to misleading results – type I and II statistical errors. We therefore evaluated the presence of outliers in the dependent and independent variables *via* Cleveland dotplots and the collinearity among the covariates. In this respect, all continuous variables were log-transformed (log x+1) to achieve homogeneity. In order to avoid the prediction of negative fitted values – meaningless from a biological point of view in terms of CFU and SH – we assumed a gamma distribution $(0, \infty;$ e.g., Abramowitz & Stegun 1972) with a log-link function. The mixed procedure allowed us to deal with temporal dependence of the samples, given that we repeated the samplings in the same plots in the two seasons. Therefore, we included the sampling plot as random factor. The regression models were fitted via the

glm. The structure of the model was y ~ DST_entrance + DST_path + DST_river + SEA.

Once we fitted this initial model, we applied model selection (Johnson & Omland 2004), in order to identify the best model structure supported by observations. We performed a backward elimination, progressively excluding variables according to AICc values (Zuur *et al.* 2009). Variables not contributing to the fit of the model – increasing the AICc value – were progressively dropped from the models thus avoiding over-fitting (Hawkins 2004). Model validation was carried out following Zuur *et al.* (2009), plotting the residuals against each covariate and the fitted values and checking for non-linear patterns in the residuals *via* the *gam* R command (Hastie 2013).

INDICATOR SPECIES ANALYSIS

In order to determine potential species to be used as indicators of disturbance within the cave, we performed Indicator Species Analysis (ISA; Dufrêne & Legendre 1997; De Cáceres & Legendre 2009). We created a community data matrix with sampling plots in rows and the CFU values of each species – proxy for abundance – in columns. We created an additional categorical variable 'site group' (*sensu* Dufrêne & Legendre 1997) grouping the samples in the three distinct classes of disturbance (see sampling design). Indicator species analysis was performed *via* the *multipatt* command in the *indicspecies* (De Cáceres & Legendre 2009) R package, which uses an extension of the original Indicator Value method (Dufrêne & Legendre 1997) by inspecting indicator species in both individual site groups and combinations of site groups (De Cáceres *et al.* 2010). The statistical significance of the relationship between indicator species and groups was tested *via* permutation test (999 permutations) with the parameter alpha set to 0.05.

RESULTS

ISOLATION AND IDENTIFICATION OF FUNGAL STRAINS

We collected 2,103 morphological taxonomic units (MTUs) of filamentous fungi on the 160 inoculated Petri dishes. The MTUs belong to 63 taxa included in Zygomycota and Ascomycota. Several yeasts and sterile mycelia were also isolated, but not identified as they fell outside the scope of our research. The latter were characterized by slow growth and they rarely developed conidiophore or sporophore. Tab. 1 reports the list of the taxonomic groups here recovered. Supporting Information 1 reports the list of the species identified on molecular base.

The most recurrent genus was *Penicillium*, followed by *Mucor*, *Trichoderma* and *Aspergillus*. It is worth noticing the occurrence of teleomorphyc species, in particular *Pseudeurotium bakerii*. Among anamorphic fungi, the most frequent filamentous fungal species were mainly saprotrophic or non-obligate parasitic, such as *Penicillium expansum*, *Aspergillus spelunceus*, *Trichoderma harzianum*, and *T. koningii*.

Tab. 1: Checklist of the species recorded in the Bossea show cave. Presence (X)/ Absence (−) of the species in each disturbance category (hp= high disturbance; mp= intermediate disturbance; lp= low disturbance) is given for winter (W) and summer (S).

Species	W-hp	W-mp	W-lp	S-hp	S-mp	S-lp
Acremonium nepalense W. Gams	−	−	−	X	−	−
Aspergillus asperescens Stolk	X	−	−	X	−	−
Aspergillus puulaanensis Jurjevic, S.W. Peterson & B.W. Horn	−	−	−	−	−	X
Aspergillus spelunceus Raper & Fennell	X	X	X	X	X	X
Aspergillus versicolor (Vuill.) Tirab.	X	X	X	−	−	X
Apergillus sp.	−	−	−	X	−	−
Bionectria ochroleuca (Schwein.) Schroers & Samuels	X	X	X	X	X	−
Cladosporium lignicola Link	X	X	X	X	X	X
Cladosporium sphaerospermum Penz.	X	X	X	−	X	−
Doratomyces sp.	−	−	−	X	X	X
Fusarium culmorum (Wm.G. Sm.) Sacc.	−	X	−	−	−	−
Geomyces sp 1	−	−	−	−	−	X
Geomyces sp 2	−	−	−	X	−	−
Gliomastix cerealis (P. Karst.) C.H. Dickinson	X	X	−	X	−	−
Mortierella alpina Peyronel	−	−	−	X	−	X−
Mucor cirinelloides Tiegh.	X	−	−	X	−	−
Mucor corticola Hagem	−	X	X	−	X	−
Mucor hiemalis Wehmer	X	X	X	−	−	X
Mucor pyriformis f. *pyriformis* Scop.	X	X	−	X	X	−
Mucor racemosus f. *racemosus* Fresen	X	X	X	X	−	−
Mucor racemosus f. *sphaerosporus* (Hagem) Schipper	X	X	X	X	X	X
Penicillium antarcticum A.D. Hocking & C.F. McRae	−	−	−	X	X	X
Penicillium atramentosum Thom	X	X	X	X	−	X
Penicillium atrovenetum G. Sm.	−	−	−	X	X	−
Penicillium bialowiezense K.M. Zaleski	−	−	−	X	−	−
Penicillium brevicompactum Dierckx	−	−	−	X	X	X
Penicillium caseifulvum F. Lund, Filt. & Frisvad	−	−	X	−	−	−
Penicillium cavernicola Frisvad & Samson	X	X	−	−	−	−
Penicillium chrysogenum Thom	X	X	X	X	X	X
Penicillium citreonigrum Dierckx	−	−	−	−	−	X
Penicillium citrinum Thom	X	X	X	X	X	X
Penicillium clavigenum Demelius	X	X	X	−	−	−
Penicillium commune Thom	X	X	−	X	−	X
Penicillium coprophilum (Berk. & M.A. Curtis) Seifert & Samson	X	X	−	−	−	−
Penicillium dendriticum Pitt	X	X	−	−	−	−
Penicillium ellipsoideospermum L. Wang & H.Z. Kong	−	−	−	−	X	−
Penicillium expansum Link	X	X	X	X	X	X
Penicillium glandicola (Oudem.) Seifert & Samson	−	X	−	−	X	−
Penicillium italicum Wehmer	X	−	−	−	−	−
Penicillium magnielliptiosporum Visagie, Seifert & Samson	−	−	−	X	−	−
Penicillium novae-zelandiae J.F.H. Beyma	−	−	−	−	−	X
Penicillium olsonii Bainier & Sartory	−	−	−	−	X	−
Penicillium phoenicum D.B. Scott & Stolk	−	−	−	X	X	−
Penicillium polonicum K.M. Zaleski	X	X	X	X	−	−
Penicillium roseomaculatum Biourge	−	X	X	−	−	−
Penicillium solitum Westling	X	−	−	X	−	X
Penicillium spinulosum Thom	−	−	X	−	−	−
Penicillium sumatrnse Svilv.	−	−	−	−	X	−
Penicillium taxi R. Schneid.	X	−	−	−	−	−

Species	W-hp	W-mp	W-lp	S-hp	S-mp	S-lp
Penicillium vulpinum (Cooke & Massee) Seifert & Samson	-	X	X	-	-	-
Penicillium sacculum C. Booth	-	-	X	-	-	-
Penicillium sp. 1	-	X	-	-	-	X
Penicillium sp. 2	X	-	X	-	-	-
Pseudeurotium bakeri C. Booth	X	-	X	-	-	-
Rhodotorula sp.	-	-	-	X	X	X
Trichocladium sp.	-	-	-	-	-	X
Trichoderma atroviridae P. Karst.	X	X	X	X	X	-
Trichoderma hamatum (Bonord.) Bainier	X	-	X	-	-	-
Trichoderma harzianum Rifai	X	X	X	-	-	-
Trichoderma koningii Oudem.	X	X	X	X	X	-
Trichoderma viridescens (A.S. Horne & H.S. Will.) Jaklitsch & Samuels	X	X	X	X	-	X
Trichoderma sp.	X	X	-	-	-	-
Sterile mycelia morphotype A	X	X	X	X	-	-
Sterile mycelia morphotype B	-	X	-	X	X	X
Sterile mycelia morphotype C	-	X	X	-	-	X
Sterile mycelia morphotype D	X	-	-	-	-	-
Sterile mycelia morphotype E	X	-	-	-	-	-
Sterile mycelia morphotype F	X	X	-	-	-	-
Sterile mycelia morphotype G	X	-	-	X	-	-
Sterile mycelia morphotype H	-	-	-	-	X	-
Yeast A	X	X	X	-	-	-
Yeast B	X	X	-	X	X	X
Yeast C	-	X	X	X	X	-
Yeast D	-	-	-	X	-	X
Yeast E	-	-	-	X	X	-

Tab. 2: Descriptive parameters of the alluvial sediment samples (hp= high disturbance; mp= intermediate disturbance; lp= low disturbance).

Season	Pressure	MTU (mean±SD)	MTU g⁻¹ (mean±SD)
Winter	lp	51.2±12.8	3.2±0.8
	mp	59.8±26.1	3.7±1.6
	hp	105.5±56.4	6.5±3.5
Summer	lp	35.8±9.3	2.2±0.5
	mp	51.5±3.6	3.2±0.2
	hp	66.8±12.9	4.7±0.8

The density of CFUs per gram of alluvial sediment was of the same magnitude order in all samples, with one only exception in winter ($11.4 * 10^6$ MTU g⁻¹). Tab. 2 reports descriptive parameters of the samples collected in each category of disturbance in the two sampling season.

REGRESSION MODELS

The most appropriate GLM structure resulting from model selection was y ~ DST_path + SEA for both Shannon-Wiener diversity (SH) and abundance (CFU). The variable distance from entrance (DST_entrance) and distance from the river (DST_river) were dropped during model selection since they did not contributed in improving the model performance. There was a decrease in Shannon diversity with increasing distances from the touristic pathway, although this trend only approached statistical significance (DST: Estimated β±SD: −0.0208±0.011, p=0.06). Diversity was also found to be significantly higher in the winter season (SEA, test relative to "Summer": Estimate β±SD: 0.1136±0.042, p<0.01; Fig. 3).

We found the same trends when modeling abundance (CFU) versus the considered covariates. Specifically, we observed a significant decrease of abundance with increasing distance from the touristic pathway (DST: Estimated β±SD: −0.1400±0.032, p<0.001) and significant higher abundance values in winter (SEA, test relative to "Summer": Estimated β±SD: 0.3055±0.098, p<0.01; Fig. 4).

INDICATOR SPECIES ANALYSIS

The indicator species analysis pointed out 3 *singletons* (*sensu* De Cáceres *et al.* 2010), representative of the
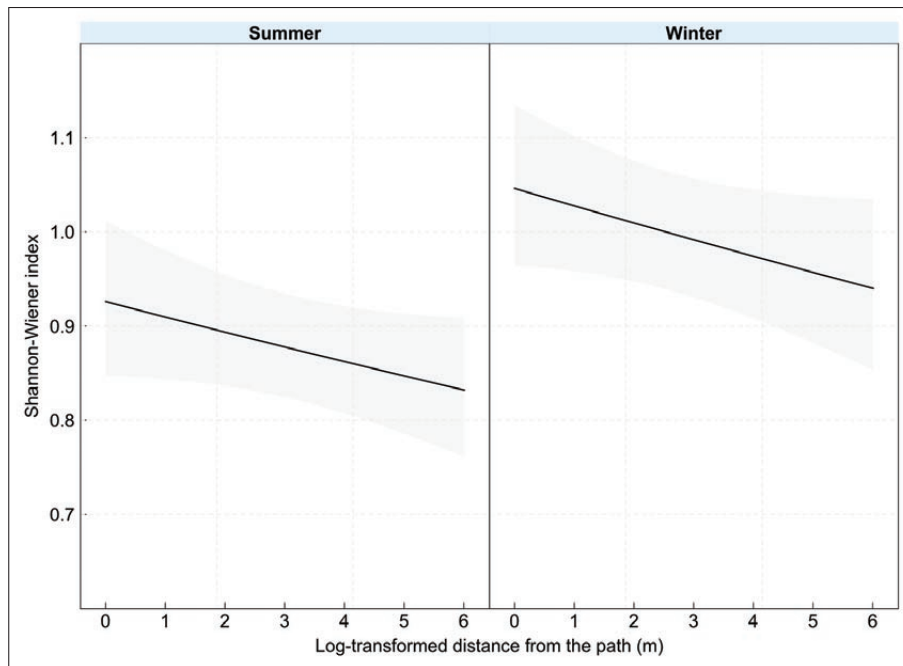
Fig. 3: Predicted values (black lines) and 95 % confidence intervals (grey surfaces) of the effect of distance from the touristic pathway (m, log-transformed) in the two sampling seasons on the diversity of the mycobiota of the alluvial sediment (Shannon-Wiener index) in the Bossea show cave. Results derived from the generalized linear models (GLM) analysis.
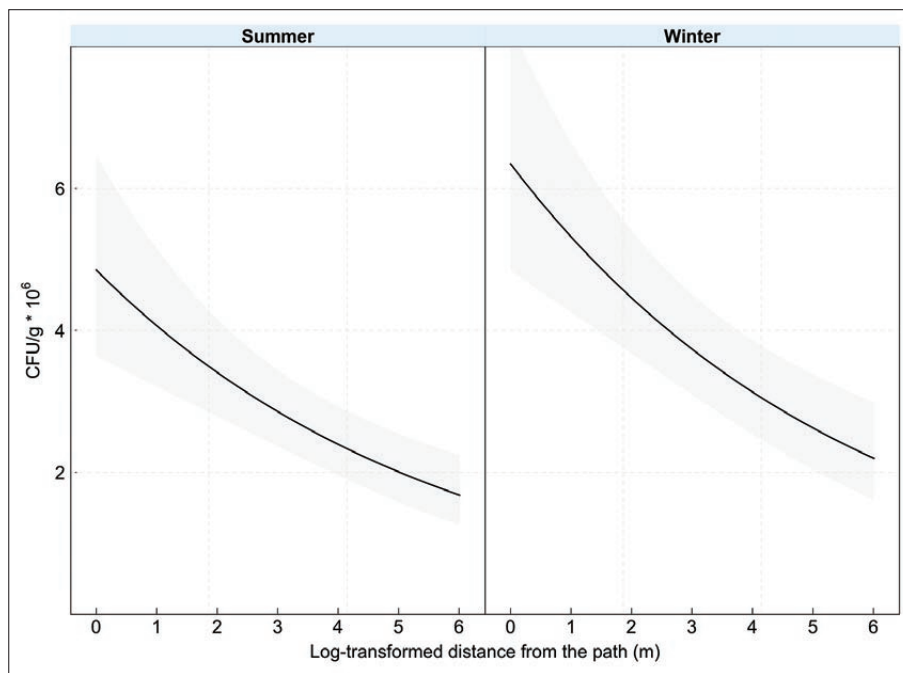

*Fig. 4: Predicted values (black lines) and 95 % confidence intervals (grey surfaces) of the effect of distance from the touristic pathway (m, log-transformed) in the two sampling seasons on the abundance of the mycobiota of the alluvial sediment (density of colony-forming unit, CFU/g * 10⁶) in the Bossea show cave. Results derived from the generalized linear models (GLM) analysis.*

3 classes of disturbance. *Aspergillus asperescens* was found to be the most suitable indicator of those plots characterized by high level of disturbance (hp; IndVal=0.577, p<0.05). The medium disturbance (mp) site group was characterized by *Mucor corticola* Hagem (IndVal= 0.608; p<0.05), whereas *Aspergillus spelunceus* was found to be indicator of low disturbance (lp; IndVal=0.787; p<0.05).

DISCUSSION

Anemochoric, hydrochoric and biochoric transportation are the natural vectors enhancing cave colonization by fungi and yeasts (Northup *et al.* 1994). If compared with non-sheltered natural habitats, diversity and biomass of fungi in caves are generally low (Vanderwolf *et al.* 2013). However, caves exploited for touristic purposes represent a remarkable exception to this general trend (e.g., Mosca & Campanino 1962; Pulido-Bosch *et al.* 1997). In fact, thanks to tourist flow, external fungal spores and yeasts access the cave ecosystems, affecting the abundance and the diversity of the subterranean microbiota (Chelius *et al.* 2009). In recent years, several authors considered the impact of tourists on hypogean microbial communities and how fungi may contribute to introduce and facilitate the proliferation of alien microorganisms inside the cave (e.g., Bastian *et al.* 2009, Jurado *et al.* 2010, Wang *et al.* 2010; Fernandez-Cortes *et al.* 2011).

In this work, we used a similar approach, focusing more specifically on alluvial sediment of the Bossea show cave. We found that alluvial sediment samples are characterized by a rich and diversified mycobiotic community (Tab. 1). The mycobiotic communities of Bossea have never been investigated before, precluding the comparison of our result with previous studies. However, in agreement with other studies conducted in similar habitats (Nováková, 2009; Fernandez-Cortes *et al.* 2011; Wang *et al.* 2010), the most recurrent genera of filamentous fungi were *Penicillium*, *Mucor*, *Trichoderma*, and *Aspergillus* and the most common species were *Penicillium expansum*, *P. brevicompactum*, and *Mucor hiemalis*. Most probably, the remarkable abundance of such species is related to their wide ecological requirements. For instance, *Penicillum expansum* and *P. brevicompactum* are cosmopolitan widespread species, which can germinate and grow at moderately cold temperature like the one found in Bossea – close to 9 °C; see Piano *et al.* (2015): p. 4, fig. 2 for a microclimatic characterization of the cave.

Diversity and abundance of fungi was found to be unevenly distributed within the cave. Regression analysis points toward a decrease of the abundance and diversity of vital propagules at increasing distances from the touristic pathway (Figs. 3-4), thus highlighting an effect of tourists in conveying microorganisms inside the cave. It is well document that tourist may release a significant amount of exogenous organic materials into the cave at every visit, in form of clothes lint, hair, skin and other kind of organic debris stuck to shoe-soles (Krajick 2001; Culver & Pipan 2009), offering suitable substrates for fungi (Dickson & Kirk 1976; Khizhmyak *et al.* 2003). As observed by Northup *et al.* (2000), this excess of organic matter often supports the proliferation of alien species to the cave mycoflora that likely compete and overwhelm the slow-growing native species. Moreover, tourists are regarded as one of the main sources of conidia and spores within the cave (Krajick, 2001), which are deposited on the surface of the alluvial sediment of the cave by air currents and gravity.

However, we acknowledge that other factors may play an additional role in conveying fungi within the cave (Vanderwolf *et al.* 2013), i.e. the anemochoric, hydrochoric and zoochoric transportation (Northup *et al.* 1994). In the specific case of Bossea, the air flowing from the entrance and the subterranean river that cross the cave may be reasonably regarded as carriers of propagules and spores, respectively for anemochoric and hydrochoric transportation. Cave-dwelling animals – such as arthropods and bats – may also play a role in this sense.

Concerning anemochoric transportation, it is worth to mention that during model validation we excluded the distance from the entrance of the cave as a significant covariate – i.e. the variable was dropped during model selection. It is reasonable to hypothesize that an effect of the local air current could have resulted in a higher presence of fungi in the nearby of the cave entrance. This trend is in contrast with several studies conducted so far (Hsu & Agoramoorthy 2001; Urzi *et al.* 2010; Kuzmina *et al.* 2012; Mulec *et al.* 2012), in which an effect of the distance from the entrance on diversity and biomass of microorganism was observed. In our case, we may hypothesize that such effect was called off by the stronger effect of the anthropogenic distance. Similar considerations apply to the hydrochoric transportation, as the distance from the river was dropped during model selection.

With respect to the biochoric transportation, it is worth to mention that the density of bat and insects are very low in this particular cave. Specifically, neither large bat colonies nor guano depositions are present in the cave. Only isolated individuals of *Rhinolophus ferrumequinum* (Rhinolophidae) and *Myotis* sp. (Vespertilionidae) are occasionally present in the cave. Moreover, we deliberately avoided areas where the presence of insects has been documented (see Morisi, 1992).

As already hypothesized by other authors (Wang *et al.* 2010, 2011), we further observed that the diversity and abundance of propagules are season dependent. Specifically, both diversity and density of colony-forming unit was higher in winter samples.

We tentatively relate this trend to the local meteorology of the cave. From a meteorological point of view, the Bossea cave is regarded as an "hot-air" trap (*sensu*

Badino 2004, 2010). Because of its ascending structure, it is affected by air convective movements, which are strictly related to the different densities of the internal and the external atmosphere. During summer the air inside the cave is colder and denser than outside, thus flowing on the floors along the conduits and exiting from the cave entrance. Cold air currents flowing on the cave floors possibly reduce the number of airborne fungi, which are conveyed outside. In parallel, a depression is created into the upper parts of the cave, causing an identical amount of outside warmer air to enter from the cave ceilings. On the other hand, in winter the internal air is warmer and less dense than outside. The airflow stops and the cave becomes a "hot-air" trap (Badino 2004, 2010). This mechanism traps airborne fungi inside the cave thus determining higher abundance in mycobiota. Although the presence of tourists is highest in summer, in winter the Bossea show cave hosts several public events (classical music concert, nativity scene) conveying in the cave a quite high number of visitors. Under this perspective, the presence of tourist may be more critical in winter.

Results obtained by Indicator Species Analysis point out certain species to be used as indicator for the degree of disturbance. According to Vanderwolf *et al.* (2013), the existence of true troglobitic fungi is uncertain. However, there are some species that have been exclusively isolated in caves. For instance the rare *Aspergillus spelunceus* was isolated few times only from hypogean environments (Raper & Fennell 1965; Marvanova *et al.* 1992). Notably, we pointed out *A. spelunceus* as indicator for low levels of disturbance and extreme conditions. The strong survival capability of *A. spelunceus* propagules is also confirmed by the ability to survive to a laboratory serial passage in peritoneal cavity of mouse (Raper & Fennell 1965).

On the other hand, results concerning medium and high disturbance indicator species – *Mucor corticola* and *Aspergillus asperescens,* respectively – have to be considered with care. For instance, the preference of *M. corticola* for intermediate disturbance seems not particularly related to its autoecology. This species is in fact a common Zygomycota in temperate zones, forest soils and swamps growing at temperatures ranging from 5 °C to 25 °C – above 30 °C there is no sporulation. *Aspergillus asperescens* is rather a rare species which resulted indicator of high disturbance. Despite its rarity, this species has been isolated not only from hypogean environments, and seems somehow related with human activity (Raper & Fennell 1965; Şimşekli *et al.* 1999); for this reason, probably, it can survive also in more competitive and disturbed situations.

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