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Ecology of *Pseudallescheria* and *Scedosporium* species in human-dominated and natural environments and their distribution in clinical samples

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This study aims to determine the occurrence of *Pseudallescheria* and *Scedosporium* species in natural and human-dominated environments. Habitats (136 sampling sites) in a transect with increasing human impact were investigated (natural areas, agricultural soils, urban playgrounds, industrial areas). Physico-chemical parameters were measured to characterize the different areas included in this investigation. Fungal identification was performed by morphology and sequence data analysis. Comparative description of virulence was largely based on the database of the ECMM/ISHAM Working Group on *Pseudallescherial Scedosporium* Infections. *Pseudallescheria* and *Scedosporium* species were most abundant in industrial areas, followed by urban playgrounds and agricultural areas. None of the species were isolated from natural habitats. The abundance of *Pseudallescheria* and *Scedosporium* species could be correlated with increasing nitrogen concentrations ($P<0.01$) and decreasing pH ($P<0.05$) within a pH range of 6.1–7.5. In general, frequency of the different *Pseudallescheria* and *Scedosporium* species in the environment is strongly enhanced by human activities, and largely differs from species distribution in clinical settings, suggesting that these species have different degrees of virulence. *Pseudallescheria boydii* is relatively frequently found as agent of human disease, while *Scedosporium dehoogii* is found almost exclusively in the environment. *Scedosporium apiospermum* is responsible for the majority of infections and is found at comparable frequency in the environment; *S. aurantiacum* and *P. minutispora* showed similar spectra, but at much lower frequencies.

Keywords *Pseudallescheria*, *Scedosporium*, ecology, species distribution, clinical samples, habitat

Introduction

Recent molecular data have shown that the opportunistic fungus *Pseudallescheria boydii* is composed of a number of individual species [1–3]. Its supposed anamorph *Scedosporium apiospermum* is now recognized as a separate taxon, and several new species such as *Pseudallescheria minutispora, Scedosporium aurantiacum* and *S. dehoogii* have been described. This unexpected diversity may have considerable consequences in the assessment of infection risks and therapy. Since the 1990s, clinicians have recognized a

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large diversity in the clinical pictures attributed to this fungal complex [4]. Several main types of infection in immunocompetent individuals are distinguished. Classically the species are known from traumatic inoculation of infectious material [5]. Infection after aspiration of contaminated water followed by a comatose period is a syndrome unique to *Pseudallescheria/Scedosporium* [6,7]. Colonization of the lungs of patients with cystic fibrosis is frequently underestimated [8]. Traumatic, as well as pulmonary routes of infection may ultimately lead to dissemination to the central nervous system [9]. Morbidity increases when patients are immunocompromised [10,11]. Given the fact that the species are common in the human-dominated environment including hospitals [12], accurate knowledge of the habitats and potential reservoirs of newly recognized species is of epidemiological significance.

Ecology of *Pseudallescheria* and *Scedosporium* species has been studied by their isolation from environmental sources and their physiology [13,14]. Authors suggested that *Pseudallescheria* and *Scedosporium* species prefer human impacted environments, such as agricultural and garden soil, sewer, polluted ponds and sediments. In addition, hydrocarbon-contaminated soil was reported as a source of these fungi [4]. *Pseudallescheria boydii* strains are able to use natural gas [15] and aromatic compounds as carbon source [16]. Growth at very low oxygen partial pressure was observed, as well as the tolerance of 5% NaCl in liquid cultures [14]. *P. boydii* was isolated from brackish and salty water, such as submerged wood in estuaria [17], tide-washed areas, and marine soil [18,19]. Ulfig [20] reported a correlation between the ammonium concentration and the occurrence of *Pseudallescheria* species in sewage sludge. From these reports, the ecological niche of *P. boydii* and *S. prolificans* was suspected to be nutrient-rich, poorly aerated habitats such as mud of eutrophic ponds or agricultural manure. However, most studies were published prior to the recently observed taxonomic diversity of *Pseudallescheria* and *Scedosporium.* Isolation procedures applied in single studies varied greatly, from inoculating soil suspensions into mice and subsequent homogenization and inoculation of organs on culture plates [21] to the use of complex media [22]. With routine isolation methods, *Pseudallescheria* and *Scedosporium* strains are easily overgrown and frequently overlooked. Hence systematically collected, quantitative data on the frequency of different *Pseudallescheria* and *Scedosporium* species in the environment are scant. Application of standardized selective isolation protocols is mandatory [23].

The present paper aims to contribute to the definition of the ecological niches of species more precisely by an exhaustive sampling campaign in different human-dominated environments, compared to natural habitats. Different soil layers were analysed, and samples were characterized chemically to obtain insights into factors possibly influencing the frequency of the fungi concerned. Data were subsequently compared to the known frequencies of species in clinical settings and the conclusions obtained from the present investigations have a significant bearing on environmental and hospital hygiene.

Materials and methods

Sampling

Sampling sites in four habitat types were chosen on the basis of presumed human impact, the lowest human influence was assumed to be in nature conservation areas. Analyzed sites were in Tyrol, Upper and Lower Austria (Table 1), where 38 sites were sampled (raised bogs in Upper and Lower Austria, montane and subalpine coniferous and mixed forests [*Picea abies, Pinus cembra, Fagus sylvatica*] on silicate [forest A] and lime [forest B] bedrock in Tyrol, Austria). In addition, a series of 62 sites was sampled in natural habitats at Vlieland, The Netherlands (Wadden Sea mud, sand dunes, North Sea beach, *Quercus* and *Pinus* forests); for these samples, no chemical analyses were performed. In environments strongly affected by human activity, 22 sites were sampled in parks, playgrounds and industrial areas in Innsbruck, Austria, and another 14 in agricultural areas of the Inn-valley, Austria, characterized by intensive cultivation. In every location, 4–6 soil samples were taken using a soil core sampler (2.8 cm in diam) within a square metre maximum, with cores collected at three depths (0–15 cm, 15–30 cm and >30 cm to a maximum of 60 cm). After removal of coarse materials (stones, roots and plant debris) the soil samples were secured in plastic bags and stored at 4°C until further processing. For determination of physico-chemical parameters the soil samples were sieved (2-mm sieve) before the analyses and the dry matter of the samples was measured.

Isolation

Ten-gram aliquots of homogenized soil samples were placed in sterilized 100-ml Erlenmeyer flasks and mixed with 40 ml sterile Tween 80, 0.01%/NaCl, 0.85% solutions (w/v). Suspensions were shaken at 300 rpm for 1 h at 37°C on a horizontal shaker. Two hundred-fifty µl of the soil suspension prepared from each sample were inoculated onto ten plates of *Scedosporium*-selective media (SceSel+) [23]. The plates were

Table 1 Characterization of the sampled habitats

		Location	Plant cover	*n**
Natural habitats	Raised bogs	'Tannermoor' and 'Karlstifter Moore' in upper and lower Austria	*Vaccinium oxycoccus, Pinus montana, Betula verrucosa, Sphagnum recurvum, S. magellanicum, Picea abies*	13
	Forests A	Central Alps in Tyrol, Austria (silicate bedrock)	Mixed forests (*Picea abies, Pinus cembra, Fagus sylvatica, Larix decidua*)	12
	Forests B	'Alpenpark Karwendel' in Tyrol, Austria (lime bedrock)	Mixed forests (*Picea abies, Pinus cembra, Fagus sylvatica, Abies alba*)	13
	Wadden island	Wadden Sea mud, North Sea beach, Vlieland, The Netherlands	Sand dunes, *Quercus* and *Pinus* forests	62
Human-dominated environments	Agricultural lands	Agricultural lands in the Inn-valley in Tyrol, Austria	Vegetable cultures and grassland	14
	Parks and playgrounds	Parks and playgrounds in Innsbruck, Tyrol, Austria	Lawns	11
	Industrial areas	Industrial areas in Innsbruck, Tyrol, Austria; sites next to streets, petrol stations and logistics zones	Lawns	11

*Number of sampling points.

incubated at 37°C for seven days, as Rainer *et al.* has demonstrated that all *Pseudallescherial Scedosporium* isolates grow under these conditions [23]. Tentative identification of all recovered colonies was accomplished by analysis of their microscopic morphology.

Quantification of ammonium

Ammonium concentrations were measured and analysed according to Kandeler [24]. Samples of 8–16 g homogenously mixed and sieved (2 mm) soil were diluted in 32–64 ml calcium chloride ($CaCl_2$) solution and extracted on an overhead shaker (50 rpm, Heidolph Reax 2, Schwabach, Germany) for 1 h. Samples were filtered and ammonium concentrations were measured with a spectrometer (wavelength 660 nm, Hitachi U-2000 spectrophotometer, Tokyo, Japan) by using Na-nitroprussid, Na-salicylate, and dichlorisocyanuracid solutions.

pH-values

Ten g homogenously mixed and sieved (2 mm) soil samples were diluted in 25 ml $CaCl_2$-solution, stirred and allowed to rest for 2 h or overnight at room temperature. After the contact time the samples were stirred again and the pH-values were measured with a pH-meter (Metrohm 744 pH-meter, Herisau, Switzerland).

Statistical analysis

Statistical analyses were performed using SPSS 15.0 (SPSS Inc., Illinois, USA). All quantitative data of the sampling were expressed as (minimum) median (maximum). Correlations between parameters were sought by the Spearman coefficient of correlation. P-values ≤ 0.05 were considered as statistically significant. In Figs. 4 and 6 mean values of CFU counts per sampling site were used.

Sequence analysis

Ten to 16 strains were isolated randomly from ten parallel plates prepared from each sample and subcultured to eliminate contaminants. The identity of the strains was determined by sequence analysis with respect to the recent systematics of *Pseudallescheria* and *Scedosporium*. DNA extraction was done as previously described [2] and amplification of the ITS-region and the β-tubulin gene was performed according to Gilgado *et al.* and Rainer and de Hoog [1,2] with the primers V9G, LS266, ITS4, ITS5, BT2-F and BT2-R. Sequence data were analysed by using Seqman II of Lasergene software (DNAStar, Wisconsin, USA) and compared using the BioNumerics package (Applied Maths, Kortrijk, Belgium).

Comparison of species distribution in clinical and environmental samples

Species distributions relative to clinical symptoms and sites, documented and compared on the basis of reference collection data, were analysed to obtain a theoretical picture of possible species relationships to pathogenicity and virulence. The comparison was calculated as the percentage of each species of all cases and involved 94 strains from the Consultant Laboratory for *Pseudallescherial Scedosporium* Infections of the Robert-Koch-Institute (RKI) Berlin and 85 strains

from the Centraalbureau for Schimmelcultures (CBS) Utrecht which were identified by ITS sequencing ($n=169$). Data are largely accessible through the website of the ISHAM/ECMM Working Group on *Pseudallescheria/Scedosporium* Infections (www.scedosporium-ecmm.com). To obtain a picture of the overall (810 strains) and habitat dependent (228 strains) frequency of *Pseudallescheria* and *Scedosporium* species, strains were identified via a research sequence database available at CBS and their occurrence was also recorded and analysed as the percentage of each species (Fig. 7).

Results

No significant differences were found in frequencies of *Pseudallescheria* and *Scedosporium* among the soil layer depths sampled ($P>0.05$; Fig. 1). The highest median density of *Pseudallescheria* and *Scedosporium* species was found in industrial areas ([0] 641 [7145] CFU g^{-1} soil dry weight (d.w.), followed by parks and playgrounds ([48] 388 [4781] CFU g^{-1} d.w.). Lowest values were observed in samples from agricultural lands ([0] 25 [853] CFU g^{-1} d.w.) (Fig. 2). No *Pseudallescheria* and *Scedosporium* strains were isolated from natural areas in Austria and The Netherlands.

The following median concentrations of ammonium (Fig. 3) were found, in decreasing order; in raised bogs (habitat type: natural areas) ([0.19] 4.11 [27.45] µg $N.g^{-1}$ d.w.), followed by forests on lime bedrock (forest B) of the same habitat type ([0.14] 0.72 [3.08] µg $N.g^{-1}$ d.w.), industrial areas ([0.02] 0.51 [2.09] µg $N.g^{-1}$ d.w.), parks and playgrounds ([0.20] 0.48 [1.91] µg $N.g^{-1}$ d.w.), forest on silicate bedrock (forest A) in natural areas ([0.11] 0.34 [2.13] µg $N.g^{-1}$ d.w.) and agricultural lands ([0.07] 0.20 [0.47] µg $N.g^{-1}$ d.w.). A positive correlation between ammonium content of soils and the density of *Pseudallescheria/Scedosporium* propagules was detectable in the combined anthropogenic habitats, even though outliers were seen in industrial areas and parks and playgrounds (Spearman $r=0.718$, $P<0.01$, $n=36$; Fig. 4). No such correlation could be found in natural areas, as no *Pseudallescheria* or *Scedosporium* strains were isolated from these environments.

Pseudallescheria and *Scedosporium* strains could be isolated from samples with a pH between 6.1 and 7.5. The pH-values (Fig. 5) were lowest in raised bogs with pH-values of (2.3) 3.3 (5.3), followed by forest A (silicate bedrock in natural areas) (2.4) 3.4 (5.4), forest B (lime bedrock in natural areas) (4.4) 6.8 (7.2), parks and playgrounds (6.1) 6.8 (7.3), industrial areas (6.4) 7.1 (7.4) and agricultural lands (7.0) 7.3 (7.5). A negative correlation between the pH-value and the abundance of the fungi for combined anthropogenic habitats was detected, even though outliers were seen in industrial areas and parks and playgrounds (Spearman $r=-0.344$, $P<0.05$, $n=36$; Fig. 6).

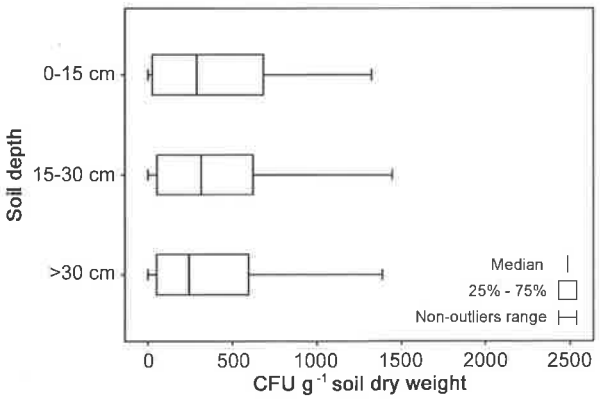

Fig. 1 Distribution of *Pseudallescheria/Scedosporium* strains in soil according to depth, layers 0–15 cm, 15–30 cm and >30 cm; outliers not shown.

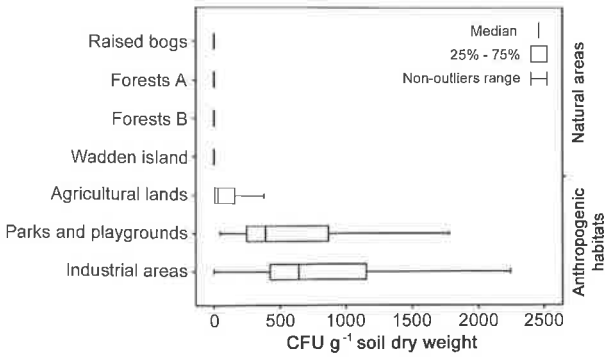

Fig. 2 Abundance of *Pseudallescheria/Scedosporium* strains in different habitat types; outliers not shown.

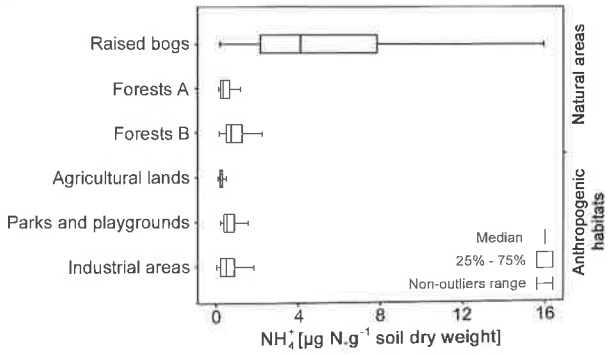

Fig. 3 Ammonium concentration (µg $N.g^{-1}$ soil d.w.) for different habitat types; outliers not shown.

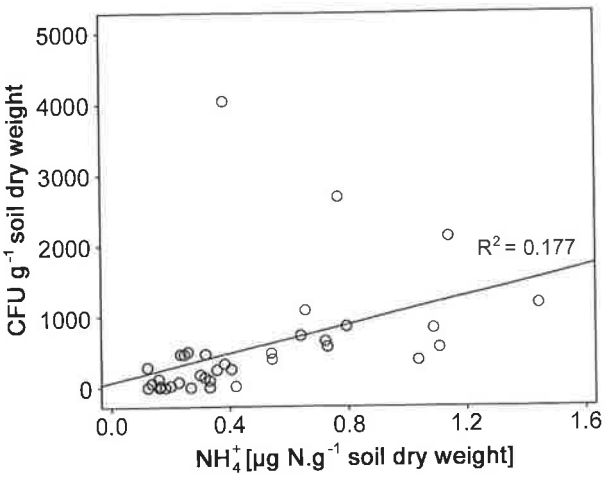

Fig. 4 Correlation between density of *Pseudallescherial Scedosporium* strains (CFU g^{-1} soil d.w.) and ammonium concentration (µg $N.g^{-1}$ soil d.w.) with regression line (Spearman r = 0.718, $P<0.01$, $n=36$).

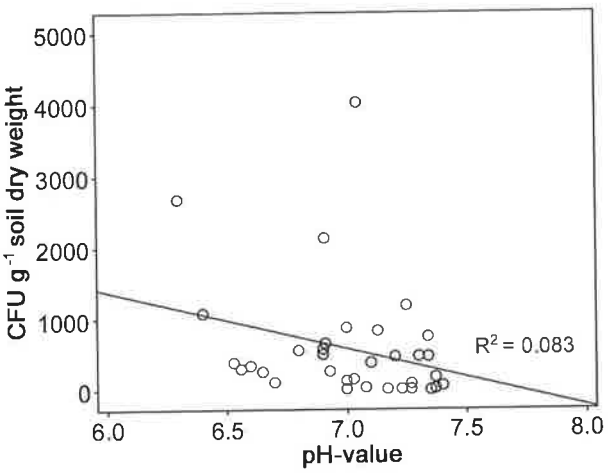

Fig. 6 Correlation between density of *Pseudallescherial Scedosporium* strains (CFU g^{-1} soil d.w.) and pH-value with regression line (Spearman r = −0.344, $P<0.05$, $n=36$).

Environmental isolation of *S. prolificans* was unsuccessful during this study – even though the isolation procedure should have been suitable [23]. *Pseudallescheria boydii* accounted for a proportion of 1.9–2.3% of the isolated *Pseudallescherial Scedosporium* strains in all habitat types. *Pseudallescheria minutispora* was isolated from industrial areas only (5.8%), *S. apiospermum* in different proportions from all antropogenic habitats (58.7% in industrial areas, 73.1% in parks and playgrounds, 77.7% in agricultural lands). A species more frequently isolated from soils than from clinical specimens was *S. dehoogii*, accounting for 28.8% in industrial areas, 16.9% in parks and playgrounds and 13.8% in agricultural lands. The decrease in the proportion of this species corresponded with decreasing ammonium concentrations in the habitat types. Relatively infrequent in environmental samples was *S. aurantiacum*, accounting for 4.8% (industrial areas) to 7.7% (parks and playgrounds) of all isolated *Pseudallescheria* and *Scedosporium* strains.

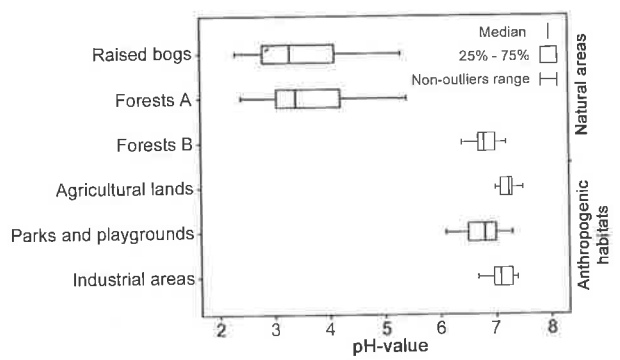

Fig. 5 pH-values in different habitat types; outliers are not shown.

The overall species distribution in a set of clinical reference strains was significantly different to the one found in the environment in the course of the present ecological study and in the general frequency (S) of the species. The data are shown as percentage stacked diagrams in Fig. 7. The largest proportion of clinical cases was caused by *S. apiospermum* (57.0%; $n=102$) and *P. boydii* (33.5%; $n=60$). Lower values were found for *S. aurantiacum* (6.1%; $n=11$) and *P. minutispora* (2.2%; $n=4$). *S. dehoogii* (1.1%; $n=2$) was isolated only from two cases of superficial infections.

Discussion

Knowledge of natural niches and reservoirs of *Pseudallescheria* and *Scedosporium* species and potential sources of infection has traditionally been based on occasional isolation of these fungi. Strains have been isolated from a wide range of environments, such as oceanic estuaria [18], frog intestines [25] and particularly from manure [26]. This suggests a natural occurrence in nutrient rich environments. Enhancement by manure suggests accumulation in fertilized agricultural soils, but also isolation from polluted environments like crude oil infested soils was reported [13]. In order to establish the frequency of *Pseudallescheria* and *Scedosporium* species correlated to human impact on the environment, a transect of soils subjected to decreasing human influence were sampled. Parameters such as ammonium concentration and pH were measured. The most striking result from this

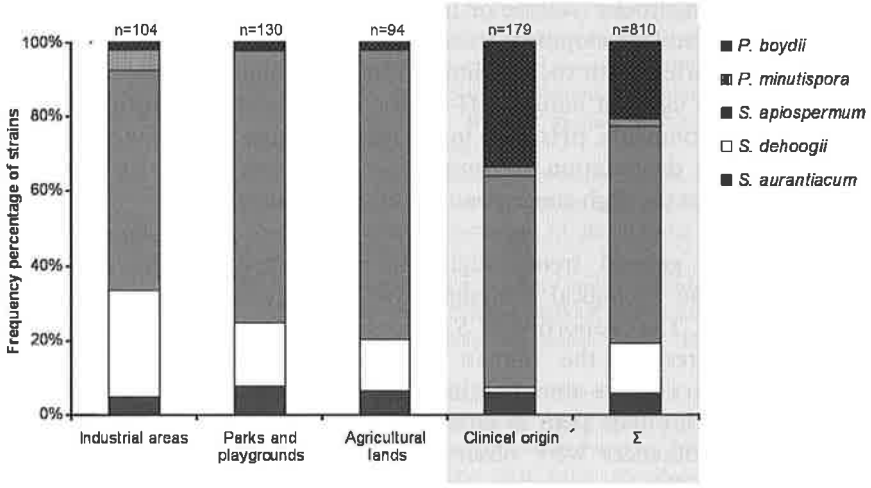

Fig. 7 Species distribution in different man-made habitats and clinical isolates. Results of database analysis are shown with grey background.

first systematic ecological investigation was that species of the genera analyzed could be isolated from human-impacted habitats only (Fig. 2). As predicted, *Pseudallescheria* and *Scedosporium* were found in agricultural soils, but frequencies were even higher in urban parks and playgrounds and in industrial areas. The sites in the latter can largely be described as having frequent construction, limited gardening and shadow, soil aggregation with adjacent sealed surfaces and emissions from traffic and street dust (rubber, road salt, hydrocarbon pollutants). Maximum values of *Pseudallescherial Scedosporium* strains were found directly adjacent to highly frequented roads and petrol stations, with up to 7,000 CFU g^{-1} d.w., as well as in a small, intensively fertilized English landscaped gardens with 4,781 CFU g^{-1} d.w. situated beside heavily used streets in the city centre of Innsbruck. Not a single *Pseudallescheria* or *Scedosporium* strain was isolated from 100 samples collected from natural habitats in Austria and The Netherlands. In an as yet unpublished follow-up study, three strains of *S. apiospermum* and one of *S. dehoogii* were derived from a natural hillside with a high number of *Rumex* plants (N-indicator) beneath a former toilet and dung heap. This again indicates high nitrogen levels as a prime requirement for growth of these fungi. This is in line with a report of Ulfig [20], who found a correlation between ammonium concentration and frequency of *Pseudallescheria*. Chemical analysis of the soil samples revealed the highest ammonium levels in raised bogs (4.11 µg $N.g^{-1}$ d.w.). An explanation for the fact that these samples did not reveal any *Pseudallescheria* is found in the acidity of that soil (pH 3.3). Chemistry of the forest soils analyzed was largely dependent on the bedrock, i.e., soils on lime bedrock with *Picea abies*, *Fagus sylvatica* or *Pinus cembra* (forests B) are mainly neutral (pH 6.8), whereas soil on silicate bedrock with *Picea abies* forests (forests A) are acidic (pH 3.4). The soils with neutral pH contained moderate amounts of nitrogen (Fig. 3), and presence of *Pseudallescherial Scedosporium* species would therefore be expected. Their absence indicates that an additional factor must be affecting the distribution and recovery of these fungi.

For human-impacted environments, the highest ammonium levels were found in industrial areas and parks and playgrounds. For these habitats, we found a positive correlation between the ammonium concentration and the abundance of *Pseudallescheria* and *Scedosporium* strains (Fig. 4). Ammonium and humic compounds are key sources of nitrogen in soil [27]. Ammonium entry into the soil is mainly due to excessive fertilization, animal husbandry systems and nitrogen emissions from the atmosphere and therefore the nitrogen contents in the sampled areas is caused by human habitation. The lower ammonium content of agricultural soils can be explained by nitrogen loss due to harvesting, erosion of uncovered areas, volatilizing nitrogen gases and pH-value, despite the fact that one would expect a high nitrogen level as a result of fertilization [27]. We hypothesize that the type of land use is a prime factor in the accumulation of *Pseudallescherial Scedosporium*. Additional factors that presumably favour the growth of members of these two genera have not yet been studied in detail but could include rising temperatures and oxygen partial pressures due to soil sealing and aggregation of soil particles in urban surroundings [28] or the presence of hydrocarbon pollutants deposited in urban soils. In contrast, a negative correlation between pH and frequency of *Pseudallescheria* and *Scedosporium* strains was found (Fig. 6). In the natural sampling areas, extreme differences in pH were noted and were ascribed

to supporting bedrocks (silicate or lime, forests A and B) and type of soil development (raised bogs). Low pH is likely to interfere with the equilibrium of NO_3^- and NH_4^+ which is usual at natural pH-values. In human-impacted environments, pHs were in the neutral range, allowing rapid degradation of ammonium. This can partially explain the high concentrations of ammonium in raised bogs.

Within this general trend, slight differences are observed in the ecological preferences of individual species (Fig. 7). The proportion of *S. dehoogii* increases with the degree of the human impact, whereas *S. apiospermum* is more abundant in agricultural soils, parks and playgrounds than in industrial areas. More remarkable differences were observed when species' distribution in the environment was compared with those observed in clinical cases. *P. boydii* is able to cause all known types of infection, and is overrepresented in cases with CNS involvement. In contrast, the species is significantly less frequent in soil. *Scedosporium apiospermum* accounts for a large proportion of clinical cases, but is comparably frequently recovered from soil. It is the second most common species responsible for CNS infections. This may allow one to infer a high virulence of this common environmental opportunist, even though laboratory studies would be required to prove this hypothesis. *Scedosporium aurantiacum* has a similar equal frequency in clinical and environmental samples, but is less frequently recovered from either of these sources. *Scedosporium dehoogii* represents the saprobic heritage of the *Pseudallescheria/Scedosporium* complex, as it was relatively frequently found in soil but only twice in mild skin infections. *Pseudallescheria minutispora* was only found in industrial areas, but it was too rare to determine its ecological preference.

We were able to show that *Pseudallescheria* and *Scedosporium* species differ in their frequency in clinical samples and in their ability to colonize man-made habitats. This study suggests that human activity promotes the presence and growth of environmental opportunists. This is of significance to understanding routes of transmission of these infectious agents. This study could serve as a cornerstone to establish *Pseudallescheria* and *Scedosporium* species as long-term indicators of environmental health and pollution, e.g., in swimming facilities and surface waters.

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