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1 **Mycosands: Fungal diversity and abundance in beach sand and recreational waters - relevance to**  
2 **human health**

3

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60

61 Abstract

62 The goal of most studies published on sand contaminants is to gather and discuss knowledge to avoid  
63 faecal contamination of water by run-offs and tide-retractions. Other life forms in the sand, however,  
64 are seldom studied but always pointed out as relevant. The Mycosands initiative was created to  
65 generate data on fungi in beach sands and waters, of both coastal and freshwater inland bathing  
66 sites. A team of medical mycologists and water quality specialists explored the sand culturable  
67 mycobiota of 91 bathing sites, and water of 67 of these, spanning from the Atlantic to the Eastern  
68 Mediterranean coasts, including the Italian lakes and the Adriatic, Baltic, and Black Seas. Sydney  
69 (Australia) was also included in the study. Thirteen countries took part in the initiative. The present  
70 study considered several fungal parameters (all fungi, several species of the genus *Aspergillus* and  
71 *Candida* and the genera themselves, plus other yeasts, allergenic fungi, dematiaceous fungi and  
72 dermatophytes). The study considered four variables that the team expected would influence the  
73 results of the analytical parameters, such as coast or inland location, urban and non-urban sites,  
74 period of the year, geographical proximity and type of sediment. The genera most frequently found  
75 were *Aspergillus* spp, *Candida* spp, *Fusarium* spp and *Cryptococcus* spp both in sand and in water. A  
76 site-blind median was found to be 89 Colony-Forming Units (CFU) of fungi per gram of sand in coastal  
77 and inland freshwaters, with variability between 0 and 6400 CFU/g. For freshwater sites, that

78 number was 201.7 CFU/g (0, 6400 CFU/g ( $p=0.01$ ) and for coastal sites was 76.7 CFU/g (0, 3497.5  
79 CFU/g). For coastal waters and all waters, the median was 0 CFU/ml (0, 1592 CFU/ml) and for  
80 freshwaters 6.7 (0, 310.0) CFU/ml ( $p<0.001$ ). The results advocate that beaches should be monitored  
81 for fungi for safer use and better management.

82

83 **Keywords:** Beach; Fungi in sand; Bathing; Allergenic fungi; Bathing water; Mycosis

84

## 85 1. Introduction

### 86 1.1 Framing fungi in sand and water

87 In 2003, the World Health Organization (WHO) published their Guidelines for safe recreational water  
88 environments, recommending that sand be looked into, especially at higher latitudes where due to  
89 lower seawater temperatures the population tends to spend less time bathing than in countries with  
90 warmer waters; but still uses beaches for all kinds of recreational purposes (WHO, 2003).

91 The Bathing Water Directive (2006/7/EC), which came into effect on the 24<sup>th</sup> of March 2006, sets  
92 tighter standards than the previous directive, although still based on the traditional faecal indicator  
93 parameters, *E. coli* and Enterococci, as recommended by the WHO's guidelines. These parameters  
94 are directly linked to the probability of a waterborne illness after bathing (WHO). Key features of the  
95 new directive include discounting of samples and establishment of a 'bathing water profile'.  
96 Microbial water quality classification is based on four-year monitoring data using 95 or 90  
97 percentiles, which are based on the probability of gastrointestinal disease due to exposure to bathing  
98 waters. Article 6 of the Bathing Water Directive (BWD) ((EU), 2006) calls for the implementation of a  
99 'bathing water profile', which results in the "identification and assessment of causes of pollution that  
100 might affect bathing waters and impair bathers health".

101 The main cause of faecal pollution in the European Union bathing waters is diffuse or non-point  
102 source pollution (Buer et al., 2018; Kataržytė et al., 2018). In contrast to point source pollution, such  
103 as combined sewage overflows, diffuse pollution usually has multiple biological and geographical

104 origins, which is therefore difficult to manage. While land run-off due to rainfall is often a major  
105 contributor, it is by no means the only contributing source. Other potentially important sources of  
106 faecal contamination impacting bathing waters and associated beaches include wildlife, in particular  
107 sea birds, dogs as well as local contaminated streams discharging onto a beach.

108 Global warming, human population (over)-growth and climate change is expected to also bring  
109 alterations of the native microbiota, due to microbial migration, coast retraction and emerging  
110 antimicrobial resistance (Weiskerger et al., 2019). These factors combined probably bring along  
111 unexpected illnesses, diagnosed and treated with some degree of difficulty by local clinicians, as  
112 addressed by Cooney in 2011 (Cooney, 2011).

113 In terms of bathing water quality research, fungi are an under-investigated biological group, and it is  
114 not represented in the BWD. However, invasive fungal infections are associated with a high rate of  
115 mortality and other ailments that we are now beginning to understand. For example, many *Candida*  
116 species frequently found in the sand are opportunistic pathogens. They are known faecal  
117 contaminants that tend to cause mucosal infections of individuals that are susceptible due to  
118 underlying medical conditions, such as diabetes or immune suppression. Babies and toddlers with  
119 their immune systems still immature, represent another at-risk group.

120 Fungal genera that have been isolated from beach sands include *Aspergillus*, *Chrysosporium*,  
121 *Fusarium* (Candan et al., 2020), *Scedosporium*, *Scytalidium*, *Scopulariopsis* (Sabino et al., 2011),  
122 *Candida* (Shah et al., 2011), *Penicillium*, *Rhodotorula* (Vogel et al., 2007), *Cladosporium*, *Mucor*,  
123 *Stachybotrys* (Bik et al., 2012; Gomes et al., 2008; González et al., 2000; Migahed, 2003),  
124 *Phialemonium* (Pong et al., 2014) and many others. *Trichophyton* and *Microsporium*, associated with  
125 skin and nail infections, also have been reported from beach sand (Sabino et al., 2011).

126 Fungal levels in beach sands may also be related to weather events (Solo-Gabriele et al., 2016). In the  
127 volcanic islands of Madeira and Porto Santo, pathogens in beach sands have been associated with  
128 intense rainfall events, flash floods, and debris flows (Marzol et al., 2006B; Romão et al., 2017a).  
129 Beach wrack (that consists of organic material) and accumulates along the coast with significant

130 amounts can also be a primary and significant source for higher fungal abundance. *Rhodotorula*,  
131 *Alternaria* and *Aspergillus* species, for example, are associated with live or decomposing aquatic or  
132 terrestrial plants or algae (Kataržytė et al., 2017; Ogaki et al., 2019).

133 The objective of the Mycosands initiative was to generate data on fungi in beach sands and waters,  
134 of both coastal and freshwater inland bathing sites.

## 135 1.2 The Fungi

### 136 1.2.1 Yeasts genera *Candida*, *Cryptococcus*, *Trichosporon* and *Geotrichum*

137 The major common human pathogenic *Candida* species include: *Candida albicans*, *Candida glabrata*,  
138 *Candida tropicalis*, *Candida parapsilosis* complex, *Pichia kudriavzevii* (formerly *Candida krusei*),  
139 *Meyerozyma guilliermondii* (formerly *Candida guilliermondii*) and rare species, such as *Candida*  
140 *dublinskiensis*. The recently emerged detected pathogen, *Candida auris*, whose ecological niche is thus  
141 far unknown, is of special interest as it shows high levels of resistance to currently available  
142 antifungal drugs (Chowdhary et al., 2017; Lockhart et al., 2017). Being an obligate human  
143 gastrointestinal tract (GIT) commensal, the presence of *C. albicans* in the environment may almost  
144 exclusively be considered as an indicator of human faecal contamination (IFC); and only occasionally  
145 from birds (Al-Yasiri et al., 2017; Angebault et al., 2013). The same may apply, partially, also to *C.*  
146 *parapsilosis*, as this complex may also be harboured in the human GIT (de Toro et al., 2011; Silva et  
147 al., 2012) and on human skin, though it is not an obligate human commensal (Cordeiro et al., 2017).  
148 *C. parapsilosis* is a major cause of invasive candidiasis in immunocompromised hosts (Trofa et al.,  
149 2008). *Candida* species may also be of environmental origins, such as *M. guilliermondii* (formerly *C.*  
150 *guilliermondii*) or *P. kudriavzevii* (formerly *C. krusei*). *M. guilliermondii* is found in polluted areas  
151 (Hirayama et al., 2018) and has been reported as causing invasive candidiasis in  
152 immunocompromised patients, exhibiting low susceptibility to two major antifungal groups: the  
153 polyenes and echinocandins (Marcos-Zambrano et al., 2017).  
154 *C. glabrata* (Pfaller et al., 2008) is also a human commensal (Hesstvedt et al., 2017) and *C.*  
155 *dublinskiensis* is a closely related species of *C. albicans*.

156 The major human pathogens of the genus *Cryptococcus* are *Cryptococcus neoformans* var.  
157 *neoformans*, *Cryptococcus neoformans* var. *grubii*, and *Cryptococcus gattii* (Chang et al., 2018; de  
158 Hoog et al., 2000b; Hagen et al., 2015; Kwon-Chung et al., 2014). The pathogenesis of cryptococcal  
159 infection involves generally respiratory infections, with a predilection to spread to the central  
160 nervous system, causing meningitis or meningoencephalitis (de Hoog et al., 2000a). Bone and  
161 cutaneous involvement are also seen. It is estimated that globally about a million cases annually  
162 occur (Maziarz and Perfect, 2016). The major susceptible human groups at risk for infection with  
163 *Cryptococcus* species are the HIV and AIDS patients, patients with hematologic malignancies and  
164 transplant patients. However, *Cryptococcus* species, particularly *C. gattii*, can cause disease also in  
165 non-compromised individuals (Rajasingham et al., 2017). *C. neoformans* var. *neoformans* and *C.*  
166 *neoformans* var. *grubii* are distributed universally, whereas *C. gattii* was thought to have a more  
167 limited geographic distribution. It has recently been reported also from non-endemic areas (Ergin et  
168 al., 2019; Rajasingham et al., 2017). In terms of ecology, all three species are environmental fungi. *C.*  
169 *neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are mainly found in environments  
170 enriched by dry bird excrements, such as pigeon droppings, while *C. gattii* is mainly found in soil  
171 associated with certain tree species, such as *Eucalyptus*, and specific animal species, such as *Pteropus*  
172 spp. (bats) and *Phascolarctos* spp. (Koalas) (Hagen et al., 2015). *Naganishia albida* has been isolated  
173 from air and has been found in water, plants and on animal skin, being reported to cause fungaemia  
174 and peritonitis (Chen et al., 2014; Choe et al., 2020; Fonseca et al., 2000; Ragupathi and Reyna, 2015;  
175 Rajasingham et al., 2017). *Papiliotrema laurentii* has also been found in soil and water (Ragupathi and  
176 Reyna, 2015), with fungaemia and meningitis cases having been reported caused by this species  
177 (Banerjee et al., 2013; Vadkertiová and Sláviková, 2006). A recent survey on fungi in the sand of  
178 Mediterranean beaches in Israel (Frenkel et al., 2020), revealed the presence of a rare *Cryptococcus*  
179 species, *Naganishia uzbekistanensis* (formerly *Cryptococcus uzbekistanensis*, which was reported  
180 (Powel et al., 2012) to cause an infection in an immunocompromised patient.



181 *Trichosporon* species are widely distributed in the environment: in soil, water, animals and are part of  
182 the human skin flora. Six species are of clinical significance; *Trichosporon asahii*, *Trichosporon*  
183 *asteroides*, *Trichosporon cutaneum*, *Trichosporon inkin*, *Trichosporon mucoides* and *Trichosporon*  
184 *ovoides* (de Almeida Junior and Hennequin, 2016). *T. inkin* and *T. asahii* cause hair infections of the  
185 scalp and groin (White piedra), which are associated with bathing in polluted water (Shivaprakash et  
186 al., 2011). *T. asahii*, has also been involved in several systemic infections (Guo et al., 2019).

187 *Geotrichum* is an ascomycetous yeast genus found worldwide in soil, water, air, sewage, plants,  
188 cereals and dairy products. It has also been found in normal human flora, sputum and faeces (Ben  
189 Neji et al., 2019). The species of clinical relevance is *Geotrichum candidum* (Durán Graeff et al.,  
190 2017). The most important risk factor for invasive infection of *G. candidum* is severe  
191 immunosuppression with neutropenia. Mortality associated with *Geotrichum*-related infections is  
192 high (Durán Graeff et al., 2017).

### 193 1.2.2 Allergenic moulds and endemic fungi

194 Fungi can affect human health in other ways as infections, including allergic reactions, irritations and  
195 toxic reactions (Fischer and Dott, 2003; Levetin et al., 2016; McGinnis, 2004).

196 Allergic reactions mainly result from sensitization and immune overreaction of the host, as suggested  
197 by clinical symptoms of rhinitis and asthma, by skin prick- and provocation tests, and with elevated  
198 blood IgE levels as a key surrogate marker of the complex host-allergen interaction (Piarroux et al.,  
199 2019).

200 Many fungal cell wall components, including chitin, glucans, mannans, mannoproteins and  
201 galactomannan, or fungal metabolites, such as enzymes and toxins, have been reported as having a  
202 causative role in allergic reactions (Douwes, 2005; Levetin et al., 2016). Furthermore, exposure to  
203 volatile organic compounds (VOC) produced by fungi growing on and degrading substrates may cause  
204 nonspecific symptoms, such as eye, nose and throat irritations, headaches and fatigue (Wålinder et  
205 al., 2005).

206 Contact allergens are mainly associated with species in two genera: *Malassezia* and *Trichophyton*.  
207 Airborne allergens are associated with a much wider variety of fungi, including *Aspergillus*,  
208 *Penicillium*, *Fusarium*, Mucorales (mainly *Rhizopus oryzae*), *Cladosporium* and *Alternaria* (for  
209 dematiaceous fungi, see below), and even yeasts.

210 Exposure to mould can cause allergic reactions in fungi-sensitized individuals, who account for about  
211 10% of the total population and 40% of patients with asthma (Burge, 2001; Mendell et al., 2011).  
212 *Aspergillus* is a life-threatening mould in immunosuppressed patient and is also responsible for the  
213 most studied and prevalent allergic fungal diseases. Allergic bronchopulmonary aspergillosis (ABPA),  
214 severe asthma with fungal sensitisation (SAFS), *Aspergillus* bronchitis and allergic *Aspergillus*  
215 rhinosinusitis account for a considerable fungal disease burden (Bongomin et al., 2017). ABPA is a  
216 severe form of the disease in atopic patients, particularly in asthmatics with a prevalence estimated  
217 from 1% to 3.5% of all asthmatic patients. It displays a higher incidence in patients with poorly  
218 controlled asthma, up to 14% (Latgé and Chamilos, 2019). In cystic fibrosis patients, *Aspergillus*  
219 sensitization is also a cause of morbidity. The high prevalence estimated between 7% to 9%  
220 underlines the importance of preventive measures in these patients (Guegan et al., 2018; Latgé and  
221 Chamilos, 2019; Michel et al., 2019). In addition to *Aspergillus*, other moulds such as *Penicillium*,  
222 *Fusarium*, *Scedosporium*, *Mucor*, *Cephalosporium*, *Verticillium* and *Chrysosporium* have also been  
223 shown to induce sensitization (Levetin et al., 2016).

224 Endemic fungi of potential interest to sand exposure are *Histoplasma*, *Coccidioides*, *Paracoccidioides*,  
225 *Blastomyces*, *Sporothrix*, *Emergomyces* and *Talaromyces marneffe*. These are soil-dwelling  
226 dimorphic fungi that are the cause of endemic fungal diseases (Ashraf et al., 2020). Exposure to an  
227 environmental reservoir in an endemic area may result in superficial but also systemic infections, the  
228 latter occurs mostly in immunocompromised patients, including AIDS patients, or solid organ  
229 transplant recipients.

### 230 1.2.3 Dematiaceous fungi

231 The ecology of black or melanised fungi is remarkably diverse. They are often described as ubiquitous  
232 organisms inhabiting mainly plant material and residing in soil (Revankar and Sutton, 2010;  
233 Wijayawardene et al., 2016). Although the majority of genera are related to plants, wood, and  
234 decaying plant material (Wijayawardene et al., 2016), several can also be isolated from air and water  
235 (Babič et al., 2017; Shelton et al., 2002), or extremophilic niches, like hypersaline environments (Zalar  
236 et al., 2019), rocks and marble (De Leo et al., 2019; Sterflinger, 2006), and also outdoor or indoor  
237 places polluted with BTEX compounds (Novak Babič et al., 2020; Prenafeta-Boldu et al., 2006). In the  
238 past, many dematiaceous fungi have been detected also in beach sand (Abdallaoui et al., 2007; de  
239 Moura Sarquis and de Oliveira, 1996; Dunn and Baker, 1984; Gomes et al., 2008; Londono et al.,  
240 2018; Migahed, 2003; Sabino et al., 2014; Salleh et al., 2018; Salvo and Fabiano, 2007; Solo-Gabriele  
241 et al., 2016; Ulfig et al., 1997; Vezzulli et al., 2009; Yee et al., 2016). Fungi, associated with beach  
242 sand belong to the plant-related genera *Alternaria*, *Chaetomium*, *Cladosporium*, *Curvularia*, *Phoma*,  
243 and *Scopulariopsis*. Other, yeast-like dematiaceous fungi like *Aureobasidium*, *Exophiala*, and  
244 *Phialophora* have rarely been isolated, the likely reason being their slow growth (Dunn and Baker,  
245 1984; Efstratiou and Velegraki, 2010; Solo-Gabriele et al., 2016). Beach sand is a specific environment  
246 that may promote the growth of melanised fungi via different factors - exposure to sun radiation,  
247 elevated humidity, strong wind, presence of salts, plants, animals, and humans (Brandão et al.,  
248 2020b; Solo-Gabriele et al., 2016). The coastline, and therefore also the sand, are from time to time  
249 exposed to adverse events, like oil or gas spills, which may additionally contribute to the presence of  
250 melanised fungi (Bik et al., 2012; Solo-Gabriele et al., 2016).

251 Although linked mainly to plants and saprophytic material, their presence in beach sand may affect  
252 the health of beach users (Brandão et al., 2020b; Solo-Gabriele et al., 2016). The most common  
253 diseases associated with sand include those of the respiratory tract, keratitis, and cutaneous and  
254 subcutaneous infections. However, also other diseases may occur from exposure to sand combined  
255 with a traumatic event. The severity depends on the extension of the trauma and host immune  
256 response (de Hoog et al., 2019; Revankar and Sutton, 2010).

#### 257 1.2.4 Dermatophytes

258 Dermatophytosis is currently a disease of global importance and a considerable public health burden,  
259 as it is estimated that 10% to 15% of the population being infected by a dermatophyte at some point  
260 during their lives (Pires et al., 2014).

261 Anthropophilic species naturally colonize humans, being transmitted between humans and causing  
262 chronic, mild, non-inflammatory infections, often reaching epidemic proportions. Geophilic  
263 dermatophytes have their reservoir in the soil around burrows of specific terrestrial mammals,  
264 feeding on keratinous debris. All animals naturally shed skin and hair, including humans. The  
265 presence of skin fragments and hairs in the soil enables the survival of these fungi (Kushwaha and  
266 Guarro, 2000). When transmitted to humans, both zoo- and geophilic species cause acute,  
267 inflammatory mycoses. Occasionally, humans infected by zoophiles remain contagious, leading to  
268 small, self-limiting outbreaks by *Microsporum canis*, *Trichophyton mentagrophytes*, *Trichophyton*  
269 *benhamiae*, *Trichophyton verrucosum*, for example, while most infections by geophilic species are  
270 quickly resolved. Due to the effectivity of human-to-human transmission, an increasing trend is  
271 observed from geophilic via zoophilic to anthropophilic (de Hoog et al., 2017).

272 The occurrence of fungi in soil can be influenced by non-biological factors such as soil temperature,  
273 humidity, rainfall, the chemical composition of the soil (including organic matter and pH) and sunlight  
274 irradiation (Coulibaly et al., 2016). Dermatophytes are no exception to this (Pontes et al., 2013).

275 Dermatophytes are important microorganisms of the soil microbiota, with cosmopolitan species and  
276 others presenting a restricted geographic distribution (Pontes et al., 2013).

277 The high prevalence of anthropophilic dermatophytes derived from dense urbanisation and  
278 increasing access to sports areas and bathing facilities (Dolenc-Voljč, 2015). Living close to animals  
279 but also living in crowded spaces or with frequent contact with soil provides multiple opportunities  
280 for disease transmission. The rise of the number of recreational areas where people may lie down  
281 (on the sand or other soils) or walking barefoot has led to an increasing concern with the possible

282 presence of dermatophytes, reported in densely populated beach sands (Efstratiou et al., 2011;  
283 Kishimoto and Baker, 1969; Müller, 1973; Sabino et al., 2011).

## 284 2. Materials and methods

### 285 2.1 Sampling sites

286 The Mycosands initiative came to be by a call for partners to join voluntarily and thus with no  
287 financial support. The sites chosen by each partner were based on selection criteria and should  
288 include: water and sand; from a coastal beach and an inland beach; of both urban and not urban  
289 areas, sampled as often and as long as possible. All partners and sites were approved by the  
290 executive committee (J. Brandão, J.P.Gagneaux and E. Segal) before enrolment.

291 The following beaches were sampled for this project by **Regions**, as shown in Figure1 (Country codes  
292 follow ISO 3166-1 definition of AU=Australia (Sydney), IE=Ireland, FR=France, GR=Greece, IL=Israel,  
293 IT=Italy, LT=Lithuania, NL=Netherlands, PT=Portugal, RO=Romania, SL=Slovenia, RS=Serbia,  
294 TR=Turkey):

295 **Black Sea** - 20 beaches - (RO): Mamaia North, Mamaia South, Constanta Modern, Constanta 3  
296 Papuci, Eforie Nord 1, Eforie Nord 2, Eforie Sud, Navodari, Costinesti 1, Costinesti 2, Venus, Neptun,  
297 Olimp, Jupiter, Cap Aurora, Mangalia 1, Mangalia 2, Saturn, 2 Mai, Vama Veche; **Mediterranean** -40  
298 beaches - (RS): River Dunav;(GR): Amorgos (Kato Akrotiri), Phaneromeni, Perani, Aeantio, Selinia,  
299 Bikini beach Alimos, Eressos;(IT):Desenzano (Garda Lake), Menaggio (Como Lake), Carate Urio (Como  
300 Lake), Pesaro (Adriatic sea), Cannero Riviera (Maggiore Lake); (TR): Belek/Kadriye, Güzelçamlı, Didim,  
301 Akbük, Ören, Akyaka, İztuzu, Sarıgerme, Alagadi, Kervansaray, Yavuz Çıkarma, Lapta, Karşıyaka,  
302 Kayalar, Devran; (IL): Ashdod, Ashkelon, Haifa, Kesaria, Palmachim, Tel Aviv; (SL): Portorož; (FR):  
303 Plage de la Lave, Plage des Catalans, Plage de l'Huveaune, Plage de la Pointé Rouge, and Plage des  
304 Goudes; **Southwest Europe** - 7 beaches - (PT): Praia da Fraga da Pegada 1 to 4, Alburrica, Carcavelos  
305 Praia Verde; **Northwest Europe** - 21 beaches - (FR): Saint-Malo (Môle beach sites 1 to 4); (IE):  
306 Sandymount, Donabate, Portrane; (LT): Melnragė, Palanga; (NL): Zandvoort, Bergen beach, River  
307 Waal (Nijmegen), Scheveningen, Kraayenbergse plassen (Cuijk), Noordwijk aan zee, Strand Blijburg,

308 Rijkerswoerdse Plas, Beach Noordwijkerhout, Nieuw-Haamstede, Beach Renesse, Beach Dishoek;  
309 **Australia** (Sydney): 3 beaches - (AU): Bondi Beach, Manly Beach, Murray Rose Pool.

310 The coordinates for each site were recorded and then mapped on Figure 1 with QGIS (Ver 3.10.0-A  
311 Coruña) which is a free licensed GIS application (GNU General Public License CC BY-SA).

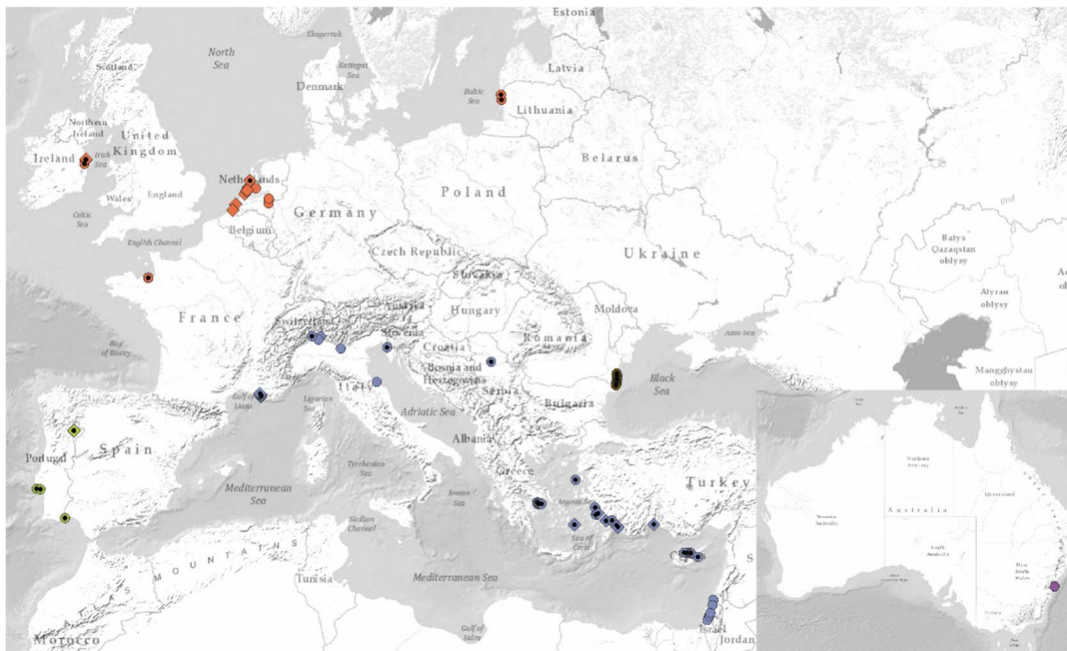
312

313 **Figure 1:** Geographical distribution of the sampling points using mapping with QGIS (Version 3.10.0-A

314 Coruña). Circles correspond to urban beaches and diamonds to non-urban beaches. Dots within the

315 shapes indicate water-sampling sites. Red=Northwest Europe, Green=Southwest Europe,

316 Blue=Mediterranean, Brown=Black Sea and Purple=Sydney (Australia)



317

318

## 319 2.2 Analytical procedures

### 320 2.2.1 Sample preparation and incubation

321 Sand: Dry (supratidal) sand samples (between 100 and 200g, between 5 and 10 cm deep) were  
322 collected aseptically (between 8 am and 12 pm) into a sterile plastic bag, labelled, and transported in  
323 a cooler to the lab as described in (Sabino et al., 2011). Official air temperatures were recorded for  
324 comparison purposes.

325 Forty grams of crude sand (not dry weight) were extracted with 40 ml of sterile distilled water by  
326 orbital shaking for 30 minutes at 100 rpm and the extract was then plated (0.2 ml) in triplicates per  
327 media (Sabouraud's Dextrose agar (SDA) and Mycosel agar (Cycloheximide, Chloramphenicol agars)).  
328 Plates were incubated for 5 days in SDA and 21 days in Mycosel agar, both at 27.5°C +/- 0.5°C.  
329 Identical colonies were counted and identified to match the different study parameters and the  
330 results were given in colony-forming units (CFU) per gram of crude sand (equivalent), as mean  
331 numbers of each triplicate.

332 Water: water samples (about 400 ml) were collected aseptically underwater (20 cm deep in 1 m deep  
333 water column between 8 a.m. and 12 a.m.), into a sterile vessel, and transported cooled (less than  
334 20°C) to the laboratory for direct plating of 0.2 ml in triplicates, after gentle shaking and as described  
335 previously, for sand.

#### 336 2.2.2 Colony counting on plates after incubation.

337 The team performed a quality control assessment based on ISO 13528:2015 to determine the  
338 consensus value of colony counting with a collective result of 95% accuracy; deemed acceptable for  
339 the group's performance.

#### 340 2.2.3 Taxonomic identification of the colonies.

341 Colonies were identified either by comparing macroscopic and microscopic features of the fungal  
342 colonies with the morphology shown in the Atlas of Clinical Fungi (de Hoog et al., 2019), and/or as  
343 described in (Frenkel et al., 2020), by the use of MALDI-ToF MS (matrix-assisted laser desorption  
344 ionization-time of flight mass spectrometry) following the manufacturer's instructions, comparing  
345 the obtained spectra against the fungal reference spectral libraries, or via molecular identification,  
346 amplifying either the primary (the Internal Transcribed Spacers (ITS 1/2) regions of the ribosomal  
347 DNA) or the secondary (the elongation factor 1 $\alpha$  (*TEF1 $\alpha$* ) or the RNA polymerase II gene (*RPB2*))  
348 fungal DNA barcodes further detailed per participating laboratory in the supplementary material,  
349 followed by the sequencing of the amplification products using the same primers. Consensus  
350 sequences were uploaded onto BLAST for taxonomic assignment. Taxonomy was only assigned for

351 sequence matches with >98 similarity and >99% query cover. All identifications followed the  
352 requisites of point 7.7 (Ensuring the validity of the results) of ISO 17025 (extended to ISO 15189 for  
353 laboratories servicing in clinical analysis), of competence of testing and calibration laboratories,  
354 which enforces many controls to ensure the validity of the results, including fungal identifications  
355 (through inter-laboratory quality assessment schemes). A table detailing the identification methods  
356 and respective quality assessment certifications is provided in the supplementary material.

357

## 358 2.3 Statistical analyses

359 Descriptive analysis was performed, using means, standard deviation, median and range (minimum  
360 and maximum) for continuous variables. The distributions of parameters were compared between  
361 urbanisation type (urban and non-urban); beach type (freshwater shores and coastal beaches); type  
362 of sand (mix and pure sand); for regions of countries aggregated by geographical and climatological  
363 proximity (Black Sea, Mediterranean, Northwest Europe, Southwest Europe and Sydney (Australia);  
364 seasons (Fall/Winter and Spring/Summer); using non-parametric tests (Wilcoxon and Kruskal-Wallis  
365 tests with Bonferroni correction for multiple comparisons). Pearson correlation coefficient was used  
366 to measure the association between parameters. Values of  $p < 0.05$  were accepted as statistically  
367 significant. All statistical analyses were performed using R version 4.0.2.

## 368 2.4 General Notes and categorisation

### 369 2.4.1 Samples

370 Sand data correspond to 372 samples from 13 different countries, collected between 2018-01-08 and  
371 2020-07-24. 38 are inland freshwater shores and 334 are coastal beaches sites; Water data  
372 correspond to 315 samples from 11 different countries, collected between 2018-01-08 and 2020-02-  
373 23. 14 are inland freshwater and 301 are coastal beach water samples. Sydney (Australia) and Israel  
374 only reported results for the sand sample and Romania did not provide detailed identification of its



375 filamentous fungi (other than for dermatophytes). Australia sampled lagged compared to the  
376 northern hemisphere for comparability purposes.

## 377 2.4.2 Parameters

378 Besides the independent analytical parameters corresponding mainly to representative species of  
379 clinical relevance found in the study, a few (**non-independent**) parameters were built to represent  
380 the less prevalent species of the study and when only the genera were identified, as follows:

381 **Yeasts** – contains all species belonging to the genera *Candida*, *Cryptococcus*, *Rhodotorula*,  
382 *Saccharomyces*, *Trichosporon* and all unidentified reports with yeast in the name (e.g. “unidentified  
383 yeast”); ***Candida* spp** – contains all *Candida* species, including all species processed independently;  
384 **‘Dermatophytes’** – contains all identifications as *Microsporum*, *Trichophyton*, *Arthroderma*,  
385 *Epidermophyton* and (unidentified) Dermatophytes; **‘Allergenic fungi’** – contains all species of fungi  
386 excluding Yeasts and Dermatophytes; **‘Dematiaceous fungi’**: contains all fungi with melanin-like  
387 pigments in the cell walls as described above, described by Brandt and Warnock (Brandt and  
388 Warnock, 2003).

389 The following independent parameters are defined hereafter: ***Aspergillus* section *Fumigati***  
390 represents *Aspergillus fumigatus sensu stricto* and all unidentified cryptic species of its section  
391 (*Fumigati*). The same applies to ***Aspergillus* section *Nigri***, ***Aspergillus* section *Flavi*** and ***Candida***  
392 ***parapsilosis*** which includes in this study represents the *sensu stricto* and its cryptic species *C.*  
393 *ortopsilosis* and *C. metapsilosis*.

## 394 2.4.3 Quantification

395 Quantification values for each fungus are displayed in CFU/g for crude sand and per millilitre for  
396 water (CFU/ml).

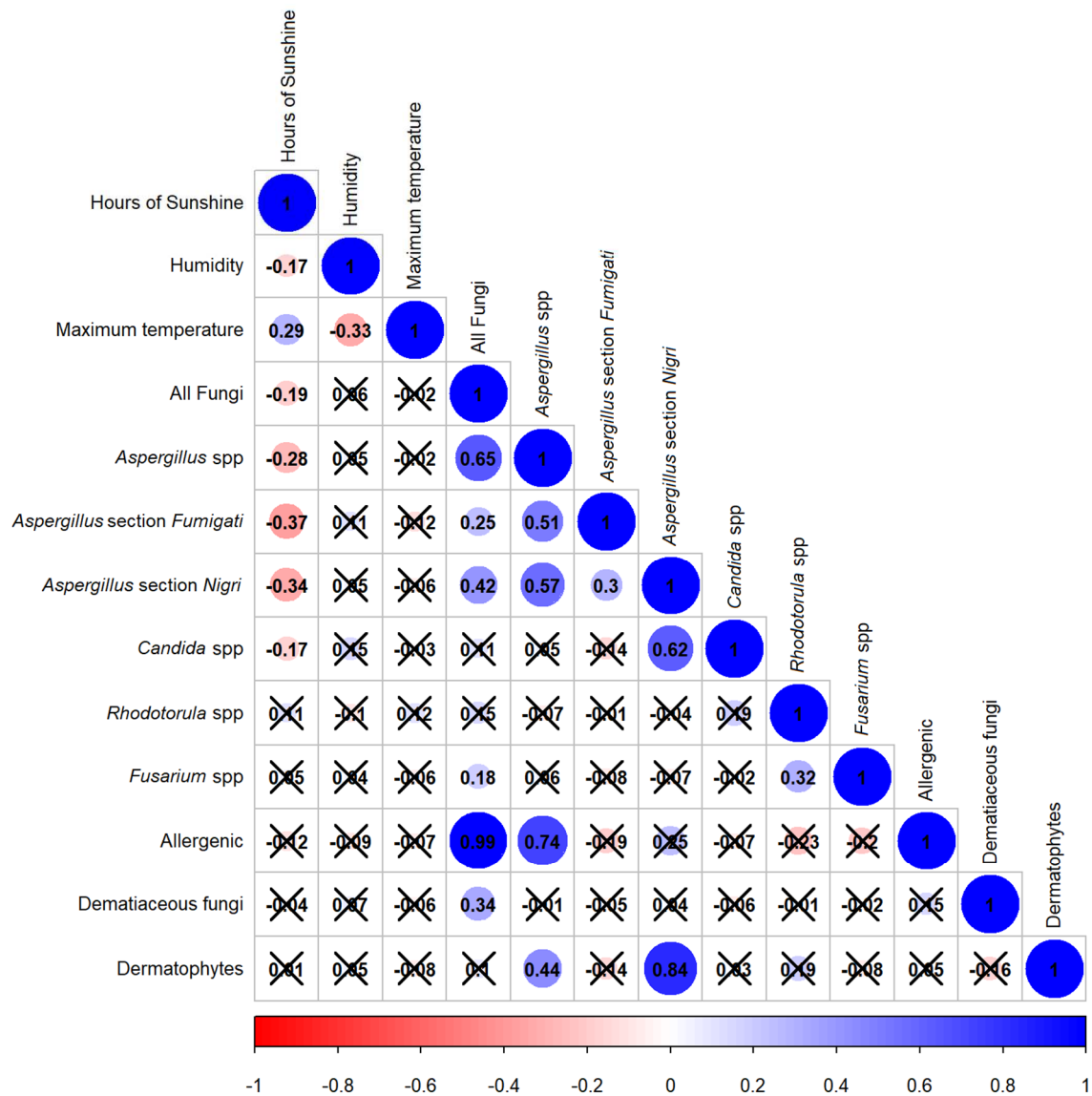
## 397 3. Results

### 398 3.1 Sand

#### 399 3.1.1 Pearson’s correlation

400 Figure 2 presents Pearson's statistically significant correlations between fungal parameters,  
401 maximum temperatures, humidity and hours of sunshine during sampling of sand. For humidity, the  
402 parameters 'All Fungi', *Aspergillus* spp., *A. section Fumigati*, *A. section Nigri* and *Candida* spp.  
403 correlate negatively to the hours of sunshine at the sampling day (-0.17, -0.19, -0.28, -0.37, and -0.34,  
404 respectively). Conversely, the maximum temperature correlates positively (0.29). Positive  
405 correlations were found between the following independent parameters: *Aspergillus* section  
406 *Fumigati*, *Candida* spp. and 'Dermatophytes' with *A. section Nigri* (0.3, 0.62 and 0.84 respectively)  
407 and *Fusarium* spp. with *Rhodotorula* spp. There were not enough pairs of data with *A. section Flavi*,  
408 *Candida albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. dubliniensis* and *Cryptococcus* spp. to  
409 estimate correlations.

410 **Figure 2:** Sand correlations between fungal parameters, maximum temperatures during sampling and  
411 hours of sunshine (statistically significant correlations do not have an X on top of the value).



412

413

414 3.1.2 Culturable mycobiota

415 The median number of fungal CFU/g in beach sand (of any kind, place, or period – All Fungi) was 89.2

416 CFU/g, with a range between 0.0 and 6400.0 CFU/g. By coastal or inland freshwater beaches, that

417 number is split into a median of 76.7 (0.0, 3497.5) CFU/g for 330 coastal beach sands and 201.7 (0.0,

418 6400.0) CFU/g for 42 inland freshwater beach sands (p= 0.010 - Table 1). Table 1 shows also that the

419 distribution of fungi between them may be quite different. The parameters *C. albicans*, *Cryptococcus*

420 spp., ‘Allergenic fungi’ and ‘Dematiaceous fungi’ appear with lower medians in coastal beach sands

421 than in freshwater beach sands, respectively 0.0 (0.0, 20.0) CFU/g to 29.2 (0.0, 50.0) CFU/g (p=0.037),  
 422 0.0 (0.0, 500.0) CFU/g to 63.3 (0.0, 110.0) CFU/g (p=0.013), 10.0 (0.0, 350.0) CFU/g to 252.5 (50.0,  
 423 6400.0) CFU/g (p<0.001) and 0.0 (0.0, 2545.0) CFU/g to 19.2 (0.0, 600.0) CFU/g (p<0.001), which  
 424 suggests a difference in the typical composition of sand culturable mycobiota. Even ‘Dermatophytes’  
 425 shows a difference, these fungi are more present in coastal beach sands ((1.7 (0.0, 150.0) CFU/g)  
 426 than in freshwater beach sands (0.0 (0.0, 166.7) CFU/g, p=0.005). The other parameters showed no  
 427 statistical significance for this comparison. *A. section Flavi* (not shown in Table 1), *C. parapsilosis*, *C.*  
 428 *tropicalis* and *C. dubliniensis* were reported only in coastal beaches.

429 **Table 1** – Comparison of results by beach types and period of the year for sand samples

Median (range) in CFU/g									
Sand									
Variable	Coastal beaches (N=330)	Fresh water beaches (N=42)	P value	Non-Urban beaches (N=86)	Urban beaches (N=286)	P value	Fall/Winter (N=128)	Spring/Summer (N=244)	P value
All Fungi	76.7 (0.0, 3497.5)	201.7 (0.0, 6400.0)	<b>0.010</b>	70.8 (0.0, 6400.0)	94.2 (0.0, 3497.5)	0.344	127.5 (0.0, 6400.0)	76.7 (0.0, 3497.5)	<b>0.016</b>
<i>Aspergillus</i> spp	5.0 (0.0, 2930.0)	13.3 (0.0, 943.3)	0.291	16.7 (0.0, 900.0)	3.3 (0.0, 2930.0)	0.176	16.7 (0.0, 2357.5)	3.3 (0.0, 2930.0)	0.071
<i>Aspergillus</i> section <i>Fumigati</i>	0.0 (0.0, 425.0)	0.0 (0.0, 66.7)	0.459	0.0 (0.0, 166.7)	0.0 (0.0, 425.0)	0.340	0.0 (0.0, 425.0)	0.0 (0.0, 91.7)	0.479
<i>Aspergillus</i> section <i>Nigri</i>	0.0 (0.0, 950.0)	0.0 (0.0, 943.3)	0.996	8.3 (0.0, 833.3)	0.0 (0.0, 950.0)	0.778	0.0 (0.0, 950.0)	0.0 (0.0, 943.3)	0.381
<i>Candida</i> spp	0.0 (0.0, 555.0)	0.0 (0.0, 50.0)	0.500	4.2 (0.0, 555.0)	0.0 (0.0, 250.0)	<b>&lt; 0.001</b>	0.0 (0.0, 555.0)	0.0 (0.0, 168.3)	0.389
<i>Candida albicans</i>	0.0 (0.0, 20.0)	29.2 (0.0, 50.0)	<b>0.037</b>	NA	0.0 (0.0, 50.0)		0.0 (0.0, 50.0)	0.0 (0.0, 50.0)	0.388
<i>Candida parapsilosis</i>	0.0 (0.0, 123.3)	NA		NA	0.0 (0.0, 123.3)		0.0 (0.0, 10.0)	2.5 (0.0, 123.3)	0.205
<i>Candida tropicalis</i>	0.0 (0.0, 41.7)	NA		0.0 (0.0, 41.7)	0.0 (0.0, 11.7)	0.189	0.0 (0.0, 35.0)	0.0 (0.0, 41.7)	0.156
<i>Candida dubliniensis</i>	1.7 (0.0, 516.7)	NA		3.3 (0.0, 516.7)	0.0 (0.0, 2.5)	<b>0.052</b>	33.3 (0.0, 516.7)	1.7 (0.0, 126.7)	<b>0.017</b>
<i>Rhodotorula</i> spp	0.0 (0.0, 1333.3)	0.0 (0.0, 60.0)	0.227	0.0 (0.0, 666.7)	0.0 (0.0, 1333.3)	0.082	0.0 (0.0, 833.3)	0.0 (0.0, 1333.3)	0.443
<i>Cryptococcus</i> spp	0.0 (0.0, 500.0)	63.3 (0.0, 110.0)	<b>0.013</b>	NA	0.0 (0.0, 500.0)		0.0 (0.0, 83.3)	0.8 (0.0, 500.0)	0.090
<i>Fusarium</i> spp	2.5 (0.0, 428.3)	0.8 (0.0, 123.3)	0.628	0.0 (0.0, 400.0)	7.5 (0.0, 428.3)	0.068	0.0 (0.0, 400.0)	8.8 (0.0, 428.3)	0.176
Yeasts	0.0 (0.0, 1333.3)	0.0 (0.0, 246.7)	0.420	1.7 (0.0, 666.7)	0.0 (0.0, 1333.3)	0.097	0.0 (0.0, 833.3)	0.0 (0.0, 1333.3)	0.510
Allergenic fungi	10.0 (0.0, 350.0)	252.5 (50.0, 6400.0)	<b>&lt; 0.001</b>	29.2 (0.0, 6400.0)	15.0 (0.0, 350.0)	0.070	68.3 (0.0, 6400.0)	14.2 (0.0, 340.0)	<b>0.006</b>
Dematiaceous fungi	0.0 (0.0, 2545.0)	19.2 (0.0, 600.0)	<b>&lt; 0.001</b>	0.0 (0.0, 600.0)	0.0 (0.0, 2545.0)	0.125	0.0 (0.0, 600.0)	0.0 (0.0, 2545.0)	0.083
Dermatophytes	1.7 (0.0, 150.0)	0.0 (0.0, 166.7)	<b>0.005</b>	0.0 (0.0, 166.7)	0.0 (0.0, 150.0)	0.820	1.7 (0.0, 166.7)	0.0 (0.0, 83.3)	0.066

430

### 431 3.1.3 Sand composition

432 The composition of the sand influences the culturable mycobiota. Pure sand has lower (All Fungi)  
 433 presence of fungi (16.7 (0.0, 300.0) CFU/g) than non-sandy shores (sediment and/or gravel) which  
 434 amounted to 90.0 (0.0, 6400.0) CFU/g (p=0.026). The other parameters, *Candida* spp., *C. albicans*, *C.*  
 435 *parapsilosis*, *C. tropicalis*, *C. dubliniensis*, *Rhodotorula* spp., *Cryptococcus* spp., Yeasts, ‘Allergenic  
 436 fungi’ and Dermatophytes, were not detected in non-sandy beaches; only ‘Dematiaceous fungi’,  
 437 though without statistical significance (0.0 (0.0, 2545.0) CFU/g for sandy beaches and 0.0 (0.0, 183.3)  
 438 CFU/g for non-sandy beaches (p=0.251). However, since these beaches were much less represented  
 439 than the sandy ones, the results of the latter parameters should not be considered as either robust  
 440 or very relevant.

441 3.1.4 Geography

442 The grouping of countries into regions (Table 2) was statistically significant only for some parameters  
 443 of sand with Sydney (Australia) showing the highest concentration of several parameters of all  
 444 regions: All Fungi (p<0.001), *A. section Nigri* (<0.001), *Candida* spp. (p=0.027), *Rhodotorula* spp.  
 445 (p<0.001), *Cryptococcus* spp. (0.0191), ‘Dematiaceous fungi’ (<0.0011), *Fusarium* spp. (< 0.001) and  
 446 the dependent parameters Yeasts (p<0.001). Nonetheless, somewhat surprisingly the hotter climates  
 447 are not necessarily the least (myco-) populated ones at the beach.

448 **Table 2** – Fungal parameters results for the sand samples by region

Median (range) in CFU/g						
Sand						
Variable	Black Sea (N=80)	Mediterranean (N=159)	Northwest Europe (N=90)	Southwest Europe (N=34)	Sydney, Australia (N=9)	P value
All Fungi	72.5 (0.5, 2170.0)	150.0 (0.0, 3365.0)	20.0 (0.0, 3497.5)	90.8 (1.7, 6400.0)	366.7 (83.3, 1533.3)	< 0.001
<i>Aspergillus</i> spp	NA	33.3 (0.0, 2930.0)	1.7 (0.0, 156.7)	1.7 (0.0, 900.0)	48.3 (0.0, 943.3)	< 0.001
<i>Aspergillus</i> section <i>Fumigati</i>	NA	0.0 (0.0, 425.0)	0.0 (0.0, 91.7)	0.0 (0.0, 16.7)	NA	
<i>Aspergillus</i> section <i>Nigri</i>	NA	0.0 (0.0, 950.0)	26.2 (2.5, 50.0)	0.0 (0.0, 833.3)	723.3 (40.0, 943.3)	0.005
<i>Aspergillus</i> section <i>Flavi</i>	NA	0.0 (0.0, 2000.0)	NA	NA	NA	
<i>Candida</i> spp	0.0 (0.0, 40.0)	0.0 (0.0, 250.0)	1.7 (0.0, 555.0)	0.0 (0.0, 21.7)	0.0 (0.0, 123.3)	0.027
<i>Candida albicans</i>	0.0 (0.0, 20.0)	0.0 (0.0, 50.0)	NA	NA	NA	
<i>Candida parapsilosis</i>	0.0 (0.0, 30.0)	0.0 (0.0, 30.0)	NA	NA	0.0 (0.0, 123.3)	
<i>Candida tropicalis</i>	NA	0.0 (0.0, 10.0)	0.0 (0.0, 41.7)	NA	NA	
<i>Candida dubliniensis</i>	NA	NA	1.7 (0.0, 516.7)	NA	NA	
<i>Rhodotorula</i> spp	0.0 (0.0, 106.0)	0.0 (0.0, 1333.3)	0.0 (0.0, 130.0)	0.0 (0.0, 3.3)	40.0 (0.0, 166.7)	< 0.001
<i>Cryptococcus</i> spp	NA	5.0 (0.0, 83.3)	0.0 (0.0, 500.0)	0.0 (0.0, 1.7)	13.3 (0.0, 110.0)	0.019
<i>Fusarium</i> spp	NA	16.7 (0.0, 428.3)	0.0 (0.0, 77.5)	0.0 (0.0, 68.3)	33.3 (6.7, 123.3)	< 0.001
Yeasts	0.0 (0.0, 106.0)	0.0 (0.0, 1333.3)	1.7 (0.0, 1265.0)	0.0 (0.0, 21.7)	123.3 (16.7, 190.0)	< 0.001
Allergenic fungi	NA	0.0 (0.0, 0.0)	15.0 (0.0, 170.0)	252.5 (50.0, 6400.0)	NA	
Dematiaceous fungi	NA	0.0 (0.0, 416.7)	0.0 (0.0, 2545.0)	15.0 (0.0, 600.0)	3.3 (0.0, 36.7)	< 0.001
Dermatophytes	NA	20.8 (0.0, 83.3)	1.7 (0.0, 53.3)	0.0 (0.0, 166.7)	NA	

449 Note: N=Number of samples

450  
 451  
 452 During this project, the median relative humidity is highest in the Northwest Europe region (78.0  
 453 (60.0, 97.0) %) and lowest in Sydney (Australia) (60.0 (60.0, 67.0) %). Sydney (Australia) is where the  
 454 highest median of CFU/g of All Fungi can be found (366.7 (83.3, 1533.3)) CFU/g; compared to 150.0  
 455 (0.0, 3365.0) CFU/g in the Mediterranean region (64.0 (19.0, 98.0) % humidity), 20.0 (0.0, 3497.5)  
 456 CFU/g, in the Northwest Europe region (78.0 (60.0, 97.0) % humidity) and 90.8 (1.7, 6400.0) in the  
 457 Southwest Europe region (70.0 (25.0, 96.0) % humidity) (Table 3). *A. section Flavi* was only reported  
 458 in the Mediterranean region, *C. dubliniensis* in the Northwest Europe region and *C. albicans* in the  
 459 Mediterranean and the Black Sea regions.

460 3.1.5 Period of the year

461 There were statistically significant differences found between the period Fall/Winter and  
462 Spring/Summer (Table 1). The parameter All Fungi shows higher counts in the Fall and Winter (127.5  
463 (0.0, 6400.0) CFU/g) compared to the Spring and Summer (76.7 (0.0, 3497.5) CFU/g),  $p=0.016$ . The  
464 'Allergenic fungi' parameter makes up for a large fraction of the All Fungi parameter, with the  
465 following data: 68.3 (0.0, 6400.0) CFU/g for Fall and Winter and 14.2 (0.0, 340.0) CFU/g for Spring  
466 and Summer ( $p=0.006$ ). Although not statistically significant, *Aspergillus* spp. also differ between  
467 period, with 16.7 (0.0, 2357.5) CFU/g and 3.3 (0.0, 2930.0) CFU/g respectively ( $p= 0.071$ ). *C.*  
468 *dublinskiensis* also contributes to the difference in periods for All Fungi with 33.3 (0.0, 516.7) CFU/g in  
469 the Fall/Winter and 1.7 (0.0, 126.7) CFU/g in the Spring/Summer (0.017).

#### 470 3.1.6 Urban versus non-urban

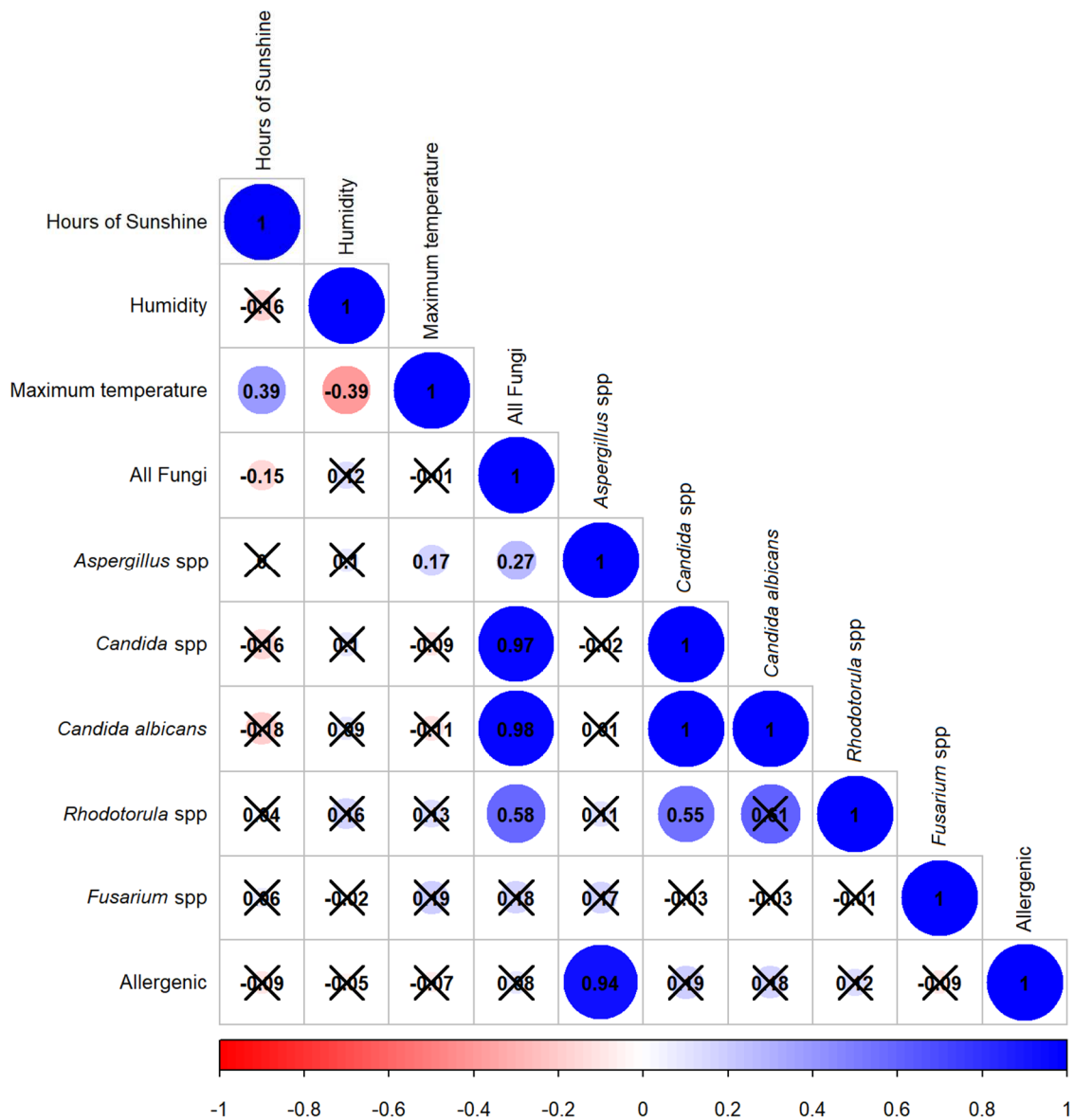
471 *Candida* spp. and *C. dublinskiensis* are more prone to non-urban environments, as shown in (Table 1),  
472 with respectively, 4.2 (0.0, 555.0) CFU/g compared to 0.0 (0.0, 250.0) CFU/g in urban environments  
473 ( $p<0.001$ ) and 3.3 (0.0, 516.7) CFU/g to 0.0 (0.0, 2.5) CFU/g ( $p=0.052$ ) in non-urban environments.  
474 *Rhodotorula* spp., *Cryptococcus* spp., *C. albicans* and *C. parapsilosis* were only detected in urban  
475 beaches. The *Rhodotorula* spp. results were 0.0 (0.0, 666.7) CFU/g in non-urban beaches, compared  
476 to 0.0 (0.0, 1333.3) CFU/g in urban beaches ( $p=0.082$ ), as medians. However, with means of 47.2  
477 CFU/g for non-urban beaches and 76.6 CFU/g for urban beaches, the very high Standard Deviations  
478 (SD) take away any strength to the original behaviour expectation (SD=160.5CFU/g and 213.7CFU/g,  
479 respectively). 'Dematiaceous fungi', for which the median provides no relevant information or  $p$ -  
480 value either when comparing non-urban and urban beaches (0.0 (0.0, 600.0) CFU/g and 0.0 (0.0,  
481 2545.0) CFU/g, respectively,  $p=0.125$ ), the means and standard deviations are respectively 53.3  
482 (133.3) and 27.2 (191.7), granting some expectation that maybe with more isolations 'Dematiaceous  
483 fungi' would possibly confirm a non-urban preference. Unlike *Fusarium* spp. which also has no  
484 statistically relevant difference (0.0 (0.0, 400.0) CFU/g for non-urban and 7.5 (0.0, 428.3) CFU/g for  
485 urban, but almost ( $p= 0.068$ ), that shows means of 28.7 (SD= 79.2) and 47.8 (SD=98.1) respectively.

#### 486 3.2 Water

487 3.2.1 Pearson's correlation

488 Figure 3 presents statistically significant correlations between fungal parameters, maximum  
489 temperatures, humidity and hours of sunshine during sampling for water. All Fungi and Yeasts  
490 correlate negatively to the hours of sunshine on the sampling day (-0.15, -0.16, respectively). The  
491 maximum temperature correlates positively with hours of sunshine with a Pearson's correlation  
492 factor of 0.39 but negatively with humidity with a factor of -0.39. Positive correlations were found  
493 between the following independent parameters: *Rhodotorula* spp. and *Candida* spp. with a factor of  
494 0.55. There were not enough pairs of data with *A. section Fumigati*, *A. section Nigri*, *A. section Flavi*,  
495 *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. dubliniensis*, *Cryptococcus* spp. and 'Dermatophytes' to  
496 estimate correlations.

497 **Figure 3:** Water correlations between fungal parameters, maximum temperatures during sampling  
498 and hours of sunshine (statistically significant correlations do not have an X on top of the value).



499

500

501 3.2.2 Culturable mycobiota

502 The median number of fungal CFU/ml of water (of any kind, place, or period – All fungi) was 0.0  
 503 CFU/g, with a range between 0.0 and 1591.7 CFU/g. The reason for this is the number of samples  
 504 with 0 CFU/ml in the pool of analyses done. Yet, comparing freshwaters and coastal waters, there is a  
 505 median number of 6.7 (0.0, 310.0) CFU/ml for freshwaters and 0.0 (0.0, 1591.7), p<0.001 for coastal  
 506 waters. Since the number of coastal waters (293) is considerably higher than freshwaters (23), and



507 mainly with low to zero CFU/ml, they pull down to 0 CFU/ml the overall medians of all beaches and  
 508 of coastal beaches (Table 3).

509 **Table 3** – Comparison of results by beach types and period of the year for water samples

Median (range) in CFU/ml									
Water									
Variable	Coastal beaches (N=293)	Fresh water beaches (N=23)	P value	Non-Urban beaches (N=86)	Urban beaches (N=286)	P value	Fall/Winter (N=113)	Spring/Summer (N=202)	P value
All Fungi	0.0 (0.0, 1591.7)	6.7 (0.0, 310.0)	< 0.001	3.3 (0.0, 1591.7)	0.0 (0.0, 310.0)	0.005	0.0 (0.0, 1591.7)	0.0 (0.0, 310.0)	0.894
<i>Aspergillus</i> spp	0.0 (0.0, 181.7)	0.0 (0.0, 201.7)	0.153	0.0 (0.0, 83.3)	0.0 (0.0, 201.7)	0.014	0.0 (0.0, 153.3)	0.0 (0.0, 201.7)	0.406
<i>Aspergillus</i> section <i>Fumigati</i>	0.0 (0.0, 90.0)	NA		0.0 (0.0, 20.0)	0.0 (0.0, 90.0)	0.008	0.0 (0.0, 20.0)	0.0 (0.0, 90.0)	0.387
<i>Aspergillus</i> section <i>Nigri</i>	0.0 (0.0, 41.7)	0.0 (0.0, 83.3)	0.865	0.0 (0.0, 83.3)	0.0 (0.0, 41.7)	0.087	0.0 (0.0, 83.3)	0.0 (0.0, 41.7)	0.440
<i>Candida</i> spp	0.0 (0.0, 1585.0)	3.3 (0.0, 8.3)	0.057	0.0 (0.0, 1585.0)	0.0 (0.0, 15.0)	0.321	0.0 (0.0, 1585.0)	0.0 (0.0, 35.0)	0.995
<i>Candida albicans</i>	0.0 (0.0, 170.0)	3.3 (0.0, 8.3)	< 0.001	0.0 (0.0, 170.0)	0.0 (0.0, 8.3)	0.348	0.0 (0.0, 170.0)	0.0 (0.0, 8.3)	0.831
<i>Candida parapsilosis</i>	6.0 (0.0, 12.0)	NA		NA	6.0 (0.0, 12.0)		12.0 (12.0, 12.0)	0.0 (0.0, 0.0)	1.000
<i>Candida tropicalis</i>	0.0 (0.0, 90.0)	NA		0.0 (0.0, 90.0)	0.0 (0.0, 1.0)	0.339	0.0 (0.0, 90.0)	0.0 (0.0, 16.7)	0.813
<i>Candida dubliniensis</i>	0.0 (0.0, 1325.0)	NA		0.0 (0.0, 1325.0)	0.0 (0.0, 2.5)	0.919	0.0 (0.0, 1325.0)	0.0 (0.0, 28.3)	0.571
<i>Rhodotorula</i> spp	0.0 (0.0, 250.0)	0.0 (0.0, 40.0)	0.280	0.0 (0.0, 25.0)	0.0 (0.0, 250.0)	0.932	0.0 (0.0, 100.0)	0.0 (0.0, 250.0)	0.932
<i>Cryptococcus</i> spp	NA	NA		NA	NA		NA	NA	
<i>Fusarium</i> spp	0.0 (0.0, 33.3)	0.0 (0.0, 10.0)	0.161	0.0 (0.0, 0.0)	0.0 (0.0, 33.3)	0.072	0.0 (0.0, 8.3)	0.0 (0.0, 33.3)	0.239
Yeasts	0.0 (0.0, 1585.0)	0.0 (0.0, 98.3)	0.188	0.0 (0.0, 1585.0)	0.0 (0.0, 250.0)	0.513	0.0 (0.0, 1585.0)	0.0 (0.0, 250.0)	0.809
Allergenic fungi	1.7 (0.0, 13.3)	5.8 (0.0, 131.7)	0.001	1.7 (0.0, 131.7)	1.7 (0.0, 26.7)	0.150	3.3 (0.0, 131.7)	1.7 (0.0, 13.3)	0.015
Dematiaceous fungi	0.0 (0.0, 111.7)	0.0 (0.0, 16.7)	< 0.001	0.0 (0.0, 33.3)	0.0 (0.0, 111.7)	0.878	0.0 (0.0, 33.3)	0.0 (0.0, 111.7)	0.006
Dermatophytes	0.0 (0.0, 1.7)	NA		0.0 (0.0, 1.7)	0.0 (0.0, 0.3)	0.661	0.0 (0.0, 1.7)	0.0 (0.0, 0.3)	0.450

510  
 511 The following parameters distribute themselves significantly between coastal and freshwaters in the  
 512 following way, respectively: *C. albicans* - 0.0 (0.0, 170.0) CFU/ml to 3.3 (0.0, 8.3) CFU/ml, p<0.001,  
 513 'Allergenic fungi' -1.7 (0.0, 13.3) CFU/ml to 5.8 (0.0, 131.7) CFU/ml, p=0.001 and 'Dematiaceous  
 514 fungi'- 0.0 (0.0, 111.7) CFU/ml to 0.0 (0.0, 16.7) CFU/ml, p<0.001. Additionally, *A. section Fumigati*, *C.*  
 515 *parapsilosis*, *C. tropicalis*, and *C. dubliniensis* and 'Dermatophytes' were only reported in coastal  
 516 beaches (Table 3).

### 517 3.2.3 Geography

518 Regarding the parameters distributed by country and by region (Table 4), there are no noticeable  
 519 regional differences in fungal parameters in bathing water, besides for *Aspergillus* spp., which show a  
 520 p-value of 0.041 for the comparison between the Mediterranean, Northwest Europe and Southwest  
 521 Europe regions only. The medians (and ranges) of this comparison are respectively 0.0 (0.0, 201.7)  
 522 CFU/ml, 0 (0.0, 181.7) CFU/ml and 0.0 (0.0, 83.3) CFU/ml and mean values (and standard deviations)  
 523 being 11.4 (31.9) CFU/ml, 3.1 (21.6) CFU/ml and 3.4 (14.9) CFU/ml. Excluding Romania, the same test  
 524 adds statistical significance for 'Dematiaceous fungi' (0.0 (0.0, 111.7) CFU/ml for the Mediterranean,

525 0.0 (0.0, 2.5) CFU/ml for the Northwest Europe and 0.0 (0.0, 21.7) CFU/ml for the Southwest Europe,  
 526 with a  $p < 0.0011$ .

527 **Table 4** – Fungal parameters results for water samples by region

Median (range) in CFU/ml					
Water					
Variable	Black Sea (N=80)	Mediterranean (N=159)	Northwest Europe (N=90)	Southwest Europe (N=34)	P value
All Fungi	0.0 (0.0, 55.0)	0.0 (0.0, 310.0)	0.0 (0.0, 1591.7)	4.2 (0.0, 131.7)	0.083
<i>Aspergillus</i> spp	NA	0.0 (0.0, 201.7)	0.0 (0.0, 181.7)	0.0 (0.0, 83.3)	<b>0.0041</b>
<i>Aspergillus</i> section <i>Fumigati</i>	NA	0.0 (0.0, 20.0)	0.0 (0.0, 90.0)	NA	
<i>Aspergillus</i> section <i>Nigri</i>	NA	0.0 (0.0, 41.7)	NA	0.0 (0.0, 83.3)	
<i>Aspergillus</i> section <i>Flavi</i>	NA	0.0 (0.0, 0.7)	NA	NA	
<i>Candida</i> spp	0.0 (0.0, 2.0)	0.0 (0.0, 12.0)	0.0 (0.0, 1585.0)	NA	
<i>Candida albicans</i>	NA	3.3 (0.0, 8.3)	0.0 (0.0, 170.0)	NA	
<i>Candida parapsilosis</i>	NA	6.0 (0.0, 12.0)	NA	NA	
<i>Candida glabrata</i>	NA	0.0 (0.0, 2.5)	0.0 (0.0, 1.7)	NA	
<i>Candida tropicalis</i>	0.0 (0.0, 1.0)	NA	0.0 (0.0, 90.0)	NA	
<i>Candida dubliniensis</i>	NA	NA	0.0 (0.0, 1325.0)	NA	
<i>Rhodotorula</i> spp	0.0 (0.0, 5.0)	0.0 (0.0, 250.0)	0.0 (0.0, 7.5)	0.0 (0.0, 25.0)	0.989
<i>Fusarium</i> spp	NA	0.0 (0.0, 33.3)	NA	0.0 (0.0, 1.7)	
Yeasts	0.0 (0.0, 10.0)	0.0 (0.0, 250.0)	0.0 (0.0, 1585.0)	0.0 (0.0, 25.0)	0.519
Allergenic fungi	NA	NA	1.7 (0.0, 13.3)	5.8 (0.0, 131.7)	
Dematiaceous fungi	NA	0.0 (0.0, 111.7)	0.0 (0.0, 2.5)	0.0 (0.0, 21.7)	<b>&lt; 0.001</b>
Dermatophytes	NA	NA	0.0 (0.0, 1.7)	NA	

528  
 529 Note: N=Number of samples

530 3.2.4 Period of the year

531 As with sand, 'Allergenic fungi' are more prevalent in the Fall/Winter (3.3 (0.0, 131.7) CFU/ml) than in  
 532 the Spring/Summer period (1.7 (0.0, 13.3) CFU/ml) with a p-value of 0.015 (Table 3).

533 3.2.5 Urban versus non-urban

534 We can find differences in the distributions of fungi in water samples by urbanisation type in the  
 535 parameters 'All Fungi' with 3.3 (0.0, 1591.7) CFU/ml for non-urban beaches and 0.0 (0.0, 310.0)  
 536 CFU/ml for urban beaches ( $p=0.005$ ); in *Aspergillus* spp. with 0.0 (0.0, 83.3) CFU/ml for non-urban  
 537 beaches and 0.0 (0.0, 201.7) CFU/ml for urban beaches ( $p=0.014$ ); and in *A.* section *Fumigati* with 0.0  
 538 (0.0, 20.0) CFU/ml for non-urban beaches and 0.0 (0.0, 90.0) CFU/ml for urban beaches ( $p=0.008$ ).

539 4. Discussion

540 Environmental fungal exposure is recognized as being associated with a range of adverse health  
 541 effects, including infectious or allergic diseases and toxic reactions. Efforts have been made in

542 standardizing methods to assess indoor fungal contamination (Méheust et al., 2013). Only limited  
543 data on sand and water contamination are available, other than the ASTM D4249-83(2005) (ASTM  
544 D4249-83(2005), 2005). *Candida* Standard, withdrawn in 2013 due to its limited use by the industry.  
545 The major strengths of the Mycosands initiative are providing a large amount of original data and the  
546 standardization of methods between thirteen countries worldwide.

#### 547 4.1 Sand

548 The results of this study, reporting a great diversity of fungi in the sand of bathing beaches, reinforce  
549 the findings of previous research (Abdallaoui et al., 2007; de Moura Sarquis and de Oliveira, 1996;  
550 Dunn and Baker, 1984; Echevarría, 2019; Efstratiou and Velegraki, 2010; Frenkel et al., 2020; Gomes  
551 et al., 2008; Kishimoto and Baker, 1969; Müller, 1973; Papadakis et al., 1997; Romão et al., 2017a;  
552 Romão et al., 2017b; Salleh et al., 2018; Tokura, 1984; Ulfing et al., 1997; Vezzulli et al., 2009; Yee et  
553 al., 2016). A variety of fungi have been identified in coastal and inland recreational beaches by other  
554 research teams (Arvanitidou et al., 2002; Di Piazza et al., 2017; Efstratiou et al., 1998;  
555 Muntañolacvetković and Ristanović, 1980; Oliveira et al., 2011; Papadakis et al., 1997; Romão et al.,  
556 2017b; Rudenko et al., 2011; Sherry et al., 1979; Vezzulli et al., 2009). We identified over 300 taxa,  
557 which is, to the best of our knowledge, and this is the largest number of taxa ever reported from  
558 beach sand and water up to now.

559 During our study, we isolated a median which is lower than what Stevens *et al.* (Stevens et al., 2012)  
560 determined, with mean populations of 109.36 CFU/g in scarcely used coastal beaches, 140.49 CFU/g  
561 in averagely used beaches and 472.29 CFU/g in heavily used beaches. Dunn & Baker (Dunn and  
562 Baker, 1984) reported from 1 - 15,900 CFU/g fungi from beach sand of Hawaii. Much smaller  
563 numbers were observed by Echevarría (Echevarría, 2019) from the sand of beaches of Costa Rica (6  
564 to 17 CFU/g), but they were isolating only filamentous fungi. Salvo & Fabiano (Salvo and Fabiano,  
565 2007) isolated 0 to 11.470 CFU/g of yeasts and 58 - 1.778 CFU/g of filamentous fungi in the  
566 northwestern Italian beaches. Migahed (Migahed, 2003) in Egypt, reported that the total count of  
567 fungi in marine sand beaches ranges between 49.7 CFU/mg to 149.93 CFU/mg. It is not easy to

568 compare and/or draw concrete conclusions concerning the abundance of fungi on beach sand, as this  
569 depends heavily on the overall pollution of the beach, the time of the year, environmental conditions  
570 such as temperature, UV radiation, precipitation events, the numbers of bathers, the grain size and  
571 the chemical nature of the sand, organic load, the presence of animals and birds. What is undeniably  
572 established by our work and that of other teams, is that the sand of bathing beaches is seeded with  
573 fungi.

574 Regarding the research published on the fungal load of sand in inland, freshwater beaches, we only  
575 identified one research article (Zatoń and Błaszczak, 2015) that isolated fungi in the sand of an urban  
576 lake beach in Poland, their numbers ranging between 1700 CFU/g and 2800 CFU/g. Considerably  
577 higher than the median abundance of all fungi observed in our project. In this area, we isolated  
578 significantly more fungi (as All Fungi numbers) from freshwater beaches than from marine beaches  
579 ( $p=0.010$ ). This could be attributed to the more favourable environmental conditions (organic load,  
580 lack of salinity, vegetation nearby). Additionally, freshwater beaches harboured in the sand  
581 significantly more *C. albicans* ( $p=0.037$ ) as well as 'Allergenic fungi' ( $p<0.001$ ), 'Dematiaceous fungi'  
582 ( $p<0.001$ ) and 'Dermatophytes' ( $p=0.005$ ). The inland beaches examined in this project were few  
583 compared to the marine beaches (little above 13%). This result may hence be seen as an indication of  
584 a trend, but more investigation would be needed to give a clearer picture.

585 Our findings on the influence of the presence of bathers on overall fungal numbers/diversity in sand  
586 suggest more isolates in urban beaches. However, the difference between urban and non-urban was  
587 not statistically significant ( $p=0.344$ ). *C. albicans*, *C. parapsilosis* and *Cryptococcus* spp. were only  
588 detected in the sand of urban beaches. Our results reinforce the findings of several research groups  
589 claiming that the presence of humans influences the numbers and species of fungi on beach sand  
590 (Bergen and Wagner-Merner, 1977; Elmanama et al., 2005; Gomes et al., 2008; Kishimoto and Baker,  
591 1969; Londono et al., 2018; Muntañolacvetković and Ristanović, 1980; Müller, 1973; Papadakis et al.,  
592 1997; Rudenko et al., 2011; Salvo and Fabiano, 2007; Stevens et al., 2012; Vezzulli et al., 2009; Vogel  
593 et al., 2007). This can be attributed to the fact that many isolates are opportunistic human

594 pathogens, easily shed into the environment. Statistically significant differences between urban and  
595 non-urban beaches were observed in the concentrations of *Candida* spp. ( $p < 0.001$ ) and *C.*  
596 *dublinsiensis* ( $p = 0.052$ ), which appeared in higher concentrations in non-urban beach sand. This may  
597 be explained by *C. dublinsiensis* having been reported to be associated with non-urban wild-life (Nunn  
598 et al., 2007).

599 Differences exist between our observations on the presence of *Rhodotorula* and the isolations of  
600 Stevens *et al.* (Stevens et al., 2012) who, investigating the correlation of human beach use to the  
601 abundance of fungi in the sand, describe *Rhodotorula mucilaginosa* and *R. sloofiae* only in marine  
602 beaches heavily used by humans, whilst our results indicate the presence of *Rhodotorula* spp in both  
603 urban and non-urban beach sand, the latter being significantly less used by people.

604 *Rhodotorula* spp. and *Cryptococcus* spp. were expected to show a statistically significant presence in  
605 urban compared to non-urban beaches, under the premise that urban beaches may sustain some  
606 level of pollution directly deriving from the urban run-off, pigeons and fossil energy hydrocarbons.  
607 Yet, only *Cryptococcus* spp. displayed that behaviour, to the extreme of not even having been  
608 detected in non-urban beaches.

609 Dermatophytes were isolated from several samples and locations, confirming their possible presence  
610 in any beach sand. Yet, no *Epidermophyton floccosum* was isolated when the bathing season ceased  
611 or from sands untouched by human foot. These data corroborate Müller (Müller, 1973), one of the  
612 earliest investigations published on pathogenic fungi in beaches, who reported isolating the  
613 dermatophyte from beach sand of the South and the North of Europe only during the bathing season  
614 (June to September). However, the epidemiology of dermatophytoses shows that this species is  
615 increasingly less present as an infectious agent (Zhan and Liu, 2017).

616 We recorded statistically significant higher counts of All fungi in the Fall/Winter period, compared to  
617 the samples from the same sampling spots in the Spring/Summer period ( $p = 0.016$ ). Significant was  
618 the differences for *C. dublinsiensis* ( $p = 0.017$ ) and 'Allergenic fungi' ( $p = 0.006$ ) also. *Aspergillus* spp.,  
619 *Cryptococcus* spp., 'Dematiaceous fungi' and 'Dermatophytes' followed this trend, although not

620 statistically significant. Previously published research results support our findings, as it has been  
621 reported that weather considerations can be important for the fungal load of sand beach. Sarquis &  
622 Oliveira (de Moura Sarquis and de Oliveira, 1996) and Assami Doi *et al.* (Doi et al., 2018) reported  
623 that in the sands of Brazilian beaches the largest number of filamentous fungi was isolated during the  
624 winter and the smallest number the summer. The contradictory results of Stevens *et al.* (Stevens et  
625 al., 2012) and Salvo & Fabiano (Salvo and Fabiano, 2007), who recorded larger numbers of yeasts in  
626 May/June/July than in September can be attributed to the fact that they did not sample during the  
627 winter. Dunn & Baker (Dunn and Baker, 1984) reported from Hawaii that in sands of comparatively  
628 high temperature (51°C) the numbers of isolated fungi were significantly fewer, than at beaches with  
629 a sand temperature of 30-35°C. Cases, where fungal populations peaked outside the bathing period  
630 have been attributed to pollution events, like a rupture of a sewage pipe (Salvo and Fabiano, 2007).  
631 In fact, that was also visible during our study with one sampling site in the Southwest region of  
632 Europe at the end of 2018, which suffered a rupture in the sewage piping facilities, resulting in the  
633 high fungal presence (September 2018) in the sand (200 CFU/g) and in the water (65 CFU/ml). The  
634 water cleared promptly upon reparation of the facilities but the sand remained contaminated for at  
635 least 3 months (105 CFU/ml and 3.3 CFU/ml in March 2019), compared to 55 CFU/g and 3.3 CFU/ml  
636 in July 2019).

637 Our results are explained by the fact that although fungi can survive a wide range of environmental  
638 conditions e.g. light, temperature and salinity (Anderson, 1979), some of these parameters can  
639 eventually become detrimental to survival for microorganisms on sand particles. Anderson  
640 (Anderson, 1979) reported that elevated seawater temperatures, as high as 35°C are considered  
641 stress conditions for the fungi and inhibit the growth of *C. albicans*, *T. mentagrophytes* and *T.*  
642 *cutaneum*. Only *Microsporium gypseum* responded positively to increased temperature. The sand  
643 habitat is severely influenced by environmental changes, particularly temperature and UV light  
644 exposure. Sand particles exposed to the sun can become very hot. When the ambient temperature is  
645 32°C, the sand temperature can be over 49°C (Cohen, 2019). Furthermore, exposure of

646 microorganisms to Ultra-violet radiation (UV) causes denaturation of proteins and damage to their  
647 genetic material. High temperatures of the sand in the summer can inhibit the growth or destroy  
648 fungi, and this combined with prolonged UV exposure in the long summer days can account for our  
649 findings.

650 Additionally, stormwater runoff, more frequent in winter than in summer, has been identified as the  
651 most frequent source of beach pollution (Dorfman et al., 2009).

652 Not all examined beaches harbour the same fungal genera/species. This holds not only for pathogens  
653 (attributed to the presence of affected humans, animals or anthropogenic interference), but also to  
654 saprophytic species. We observed the greatest variety of fungi in the Mediterranean region.  
655 Northwest European beach sands exhibited a slightly larger diversity of fungal populations than  
656 Southwestern European regions (Table 2). These observations are supported by Dunn & Baker (Dunn  
657 and Baker, 1984); Gomes *et al.* (Gomes et al., 2008); Salvo & Fabiano (Salvo and Fabiano, 2007), who  
658 noted that although the beaches they examined shared several species, each beach had species  
659 unique to it.

660 Considering the expectations surrounding the ecological niches of dematiaceous fungi, Sydney  
661 (Australia) shows similarities with inland water catchments because of being located in a river mouth  
662 and thus inevitably populated with vegetable matter originating upstream in the river. The site  
663 Murray Rose Pool, being situated inland compared to the other two sites, has twice as many genera  
664 of black moulds; this might explain the contrast of their results with other urban sampling sites.  
665 Urban or not urban might thus not be as much of an influencing variable on 'Dematiaceous fungi's as  
666 initially thought. In fact, the following taxa were isolated during this study: *Alternaria*, *Arthrimum*,  
667 *Aureobasidium*, *Bipolaris*, *Chaetomium*, *Cladophialophora*, *Cladosporium*, *Coniochaeta*, *Curvularia*,  
668 *Didymella*, *Epicoccum*, *Exophiala*, *Exserohilum*, *Fonsecaea*, *Hortaea*, *Madurella*, *Microsphaeropsis*,  
669 *Neopyrenochaeta*, *Paraphoma*, *Phaeoacremonium*, *Phialophora*, *Phoma*, *Pseudallescheria*,  
670 *Pyrenochaeta*, *Robillarda*, *Scopulariopsis*, and *Sporothrix*. Their presence was more noticeable in

671 freshwater settings given the proximity of vegetation, which provides a carbon source for their  
672 growth.

673 Despite the relatively large scale of this study, the presence and composition of fungal mycobiota  
674 may be site-dependent, since many sites exhibit very low numbers of fungal presence and others,  
675 quite the opposite. This renders modelling of sand culturable mycobiota relatively difficult for any  
676 site without supporting historical data. Values presented in this study should thus serve only as a  
677 location-blind starting point for more geographically focused studies with similar aims and scopes.

## 678 4.2 Water

679 The results of the water analysis indicate an interest in determining fungi in water, especially in  
680 freshwater, given the opportunistic and almost exclusive human faecal natures of *C. albicans* and the  
681 intimate contact with bathers, as mentioned in the introduction. Under this premise, faecal indicator  
682 bacteria (FIB) currently used in bathing water quality regulation - *E. coli* and *enterococci* ((EU), 2006)  
683 may not be used in this study, which focuses on fungi only, but our results show potential human  
684 faecal pollution in 45 of the 302 coastal bathing waters (15%) and 4 of the 14 bathing freshwaters  
685 (28.6%).

686 Our results reveal a great diversity of fungi in the water of the beaches examined throughout Europe.  
687 These findings are in agreement with previous research, reporting a variety of filamentous fungi and  
688 yeasts from marine beaches (Arvanitidou et al., 2002; Aulicino et al., 2001; Gomes et al., 2008;  
689 Loureiro et al., 2005; Maciel et al., 2019; Mates, 1994; Oliveira et al., 2020; Oliveira et al., 2011; Roth  
690 Jr et al., 1962; Velegraki-Abel et al., 1987; Vezzulli et al., 2009). From freshwater beaches, there are  
691 also reports of considerable diversity in fungi isolations (Biedunkiewicz and Góralaska, 2016; Brandão  
692 et al., 2010; Falcão et al., 1993; Góralaska et al., 2020; Kiziewicz et al., 2004; Sherry et al., 1979; Wójcik  
693 et al., 2003).

694 Whilst several research projects have dealt qualitatively with the issue of fungi in recreational  
695 waters, publishing on the diversity and the taxa detected, little was published on the abundance of  
696 isolated fungi. We isolated significantly more fungi (measured as 'All fungi') from freshwater than



697 from coastal waters ( $p < 0.001$ ). This could be attributed (as in the case of sand) to environmental  
698 conditions being more favourable to their survival and growth in freshwater bodies than the sea  
699 (organic nutrients, lack of salinity, vegetation). The freshwaters of the inland beaches harboured  
700 significantly more *C. albicans* ( $p < 0.001$ ), 'Allergenic fungi' ( $p = 0.001$ ) and 'Dematiaceous fungi'  
701 ( $p < 0.001$ ). More specifically we detected from 0 to 1592 CFU/ml of All fungi from coastal waters  
702 (mean 0 CFU/ml). Velegraki-Abel *et al.* (Velegraki-Abel *et al.*, 1987) isolated 30-1020 CFU/50ml yeasts  
703 from marine beach water, Aulicino *et al.* (Aulicino *et al.*, 2001) from  $1 - 10^3/100\text{ml}$  yeasts. From  
704 inland beach waters, we isolated from 0 to 310 CFU/ml of All fungi (mean 6.7 CFU/ml). Similar  
705 findings were published by Wójcik *et al.* (Wójcik *et al.*, 2003) who isolated from 8 to 32800  
706 CFU/100ml yeasts from reservoir water used for recreational bathing. Goralska *et al.* (Góralaska *et al.*,  
707 2020) detected an average of 40 to 460 CFU/100ml in recreational freshwater ponds in Poland.

708 The presence of bathers appears to affect the abundance of fungi isolated from beach water. Urban  
709 beaches have significantly more fungi (measured as All fungi) than non-urban beach water ( $p = 0.005$ ).  
710 *Aspergillus* spp. and *Aspergillus* section *Fumigati* are the two fungal groups that show significantly  
711 higher numbers in urban beaches ( $p = 0.014$  and  $p = 0.008$  respectively). Similar results have been  
712 reported for yeasts. Papadakis *et al.* (Papadakis *et al.*, 1997) claim that yeasts of human origin  
713 correlated with the numbers of swimmers in seawater. Velegraki-Abel *et al.* (Velegraki-Abel *et al.*,  
714 1987), also investigating in Greek coastal bathing waters, reported that yeast counts increased during  
715 the summer. Sherry *et al.* (Sherry *et al.*, 1979) published that maximum numbers of *C. albicans* were  
716 observed in association with peak bather loads at the beaches of a lake in Canada.

717 Different seasons appeared to affect significantly the numbers of fungi we isolated from water only  
718 in the case of 'Allergenic fungi' ( $p = 0.015$ ) and 'Dematiaceous fungi' ( $p = 0.006$ ), who were more  
719 abundant in the Fall/Winter period as compared to the Spring/Summer period. In all other  
720 categories, the differences were not statistically significant. Data discussing differences in fungal  
721 isolation from bathing waters have not been published, to the best of our knowledge, except for  
722 Goralska *et al.* (Góralaska *et al.*, 2020) who reported, in inland bathing ponds, the highest diversity and

723 abundance of filamentous fungi in June, just before the bathing season, compared to the period July  
724 – September.

725 The geographical profile of fungal taxa we isolated from recreational waters is similar to that of fungi  
726 isolated from the sand: the greatest variety appeared in the Mediterranean region, the smallest in  
727 the Black Sea bathing waters. Northwest European beach waters presented a slightly larger diversity  
728 of fungal populations than the Southwest (Table 4).

729 The statistical analysis of the water samples suggests that the amount of inoculum used in this study  
730 (less than 1 ml per sample) was not enough to produce a robust overview of fungal contaminants in  
731 water. Filtering 100 ml, as for drinking water, leads to too many colonies in a small filter (personal  
732 experience of one of the authors with coastal bathing waters - J. Brandão). Extending this study with  
733 more sampling events and replicas might resolve this problem. Alternatively, filtering up to 5 ml (25x  
734 more sample than for each replica of this study) might also help produce more data to generate  
735 more data.

#### 736 4.3 GENERAL COMMENTS TO BOTH WATER AND SAND

737 *A. section Fumigati* and *A. niger sensu lato* constituted a major portion of the isolates in the  
738 Mediterranean (present in 10 samples) whereas *Penicillium* spp. dominated (present in 9 samples) in  
739 the Southwest region. *Rhodotorula* and *Candida* species were the truly ubiquitous ones isolated from  
740 every region. *C. albicans* is the yeast most often isolated from marine and freshwater beaches by  
741 other researchers (Arvanitidou et al., 2002; Aulicino et al., 2001; Biedunkiewicz and Góralaska, 2016;  
742 Gomes et al., 2008; Loureiro et al., 2005). *Rhodotorula* was reported in higher numbers in two cases  
743 (Velegraki-Abel et al., 1987; Wójcik et al., 2003), but these research groups reported *C. albicans*  
744 isolations amongst the most common species, too.

745 Valdes-Collazo *et al.* (Valdes-Collazo et al., 1987) found that *C. albicans* survives well in freshwater  
746 and marine water, so bathing in its presence necessarily represents an exposure setting to this  
747 organism were intimate contact with the bather's skin and mucosae take place. Conversely, this  
748 species does not respond well to dehydration, which renders it to be a possible indicator of recent

749 human faecal pollution in the sand and in water; its decay is similar to that of *E. coli*, as noted by  
750 Kashbur *et al.* (Kashbur *et al.*, 1980).

751 Given their allergenic nature, *Penicillia* were considered in this study solely as part of the parameter  
752 'Allergenic fungi' statistical strength, although the authors recognize that with high counts, aerosols  
753 might trigger allergic reactions in susceptible patients.

754 The prediction of pathogen risks is essential to beach management. The increase of chances of an  
755 introduction of fungal pathogens into the population should be avoided, because of the increased  
756 numbers of immunocompromised people, the advances in chemotherapeutic treatments that allow  
757 patients to move around and visit recreational spots, as well as the ageing of the human population.  
758 Identifying the most important routes of fungal transmission in the sand would allow beach  
759 management officials to improve services and reduce risks of exposure to pathogenic fungi. Little  
760 research has been published on within-one-beach differences in fungal populations (Brandão *et al.*,  
761 2020a; Velegraki *et al.*, 2012) providing evidence that sand around showers exhibits the highest  
762 numbers of keratinophilic isolates, followed by sand from children's playground areas and from  
763 sports activities areas. Should such findings be supported by further research it would be possible for  
764 beach managers to improve the management of shower run-offs or the treatment of shower  
765 effluents or relocate showers in order to avoid spread/unnecessary contact.

766

## 767 5. Conclusions

768 Traditionally, microbial safety regulation of beaches is based on the exposure results of a general  
769 population (WHO). However, fungi need to be addressed differently: many of the fungi found in this  
770 study are the cause of fungal ailments in susceptible beach users; both in the sand and in water.  
771 Regulation should thus enforce their detection in order to advise these users of the probable  
772 exposure.

773 Fungi are nature's organic matter recycling machines. Different species may thrive mainly with  
774 specific substrates, rendering them good indicators for specific situations. The presence of *Fusarium*

775 species in the sand, for example, may indicate remains of vegetable debris, as these species are plant  
776 pathogens and colonisers. Another group highly associated with densely vegetated areas are the  
777 Melanised fungi. These include species that cause deep and often lethal infections, as well as  
778 allergies. A list of the genera found in this study can be found in the supplementary material.

779 The current absence of a dose-response on fungal ailments, quantitative mycological risk assessment  
780 or any epidemiological study, hinders possible regulatory plans based on the health implications of  
781 exposure to fungi at the beach. The current study, involving the assessment of culturable mycobiota  
782 in a variety of geographic areas and climatic conditions, might contribute to the formation of a broad  
783 view on its relevance to human health. A median of site-blinded total fungal of 89 CFU/g was found  
784 in this study and could be used as a reference for beaches with no historical analytical data (in  
785 Europe and possibly in other geographical areas).

786 It is the opinion of the authors that the monitoring of fungi in beach sand and water is relevant,  
787 particularly for the most susceptible beach users. The authors of this study also consider that at least  
788 *C. albicans* and dermatophytes should also be monitored as additional health-oriented parameters  
789 (besides total fungal colony count). Other possible fungal parameters, species or genera should be  
790 considered on a need-to-do basis, according to persistent pollution or specific health endemic  
791 requirements.

792 More work needs to be done on water. Further research will more comprehensively characterise the  
793 water fungal contaminants better and allow a better assessment of possible future regulatory  
794 parameters.

795

## 796 Highlights

- 797 - Fungi are missing from water and sand health protection regulatory parameters.
- 798 - Both sand and water should be monitored for fungi
- 799 - The median value of 89 CFU/g of all fungi may serve as a reference for sand regulation
- 800 - *Candida albicans*, dermatophytes, endemic fungi and other fungi should be considered

801 - Fungal analysis of water needs more data before reference values can be established

802

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814

### 815 Declaration of interests

816 The authors declare that they have no known competing financial interests or personal relationships  
817 that could have appeared to influence the work reported in this paper.

818

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851

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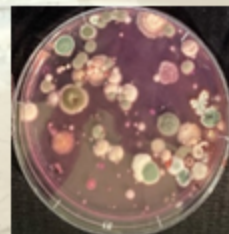
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Sand Sample



Primary Isolation



Single Colony

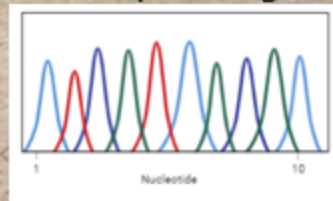
MALDI-TOF

Macroscopic and  
Microscopic ID

Fungal DNA Barcode Amplification  
(ITS1/2, *TEF1α* and/or *RPB2*) and  
other *taxa*-specific sequences

Comparison to Reference  
Spectral Database for ID

Sequencing



**Fungal characterisation of beach sand and recreational  
waters (species and CFU/g & CFU/ml)**

BLAST to Reference  
Sequence Databases for ID