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

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Abstract

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

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 CFU/g). For coastal waters and all waters, the median was 0 CFU/ml (0, 1592 CFU/ml) and for freshwaters 6.7 (0, 310.0) CFU/ml ($p<0.001$). The results advocate that beaches should be monitored for fungi for safer use and better management.

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

1. Introduction

1.1 Framing fungi in sand and water

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

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

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

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

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

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

Fungal genera that have been isolated from beach sands include *Aspergillus*, *Chrysosporium*, *Fusarium* (Candan et al., 2021), *Scedosporium*, *Scytalidium*, *Scopulariopsis* (Sabino et al., 2011), *Candida* (Shah et al., 2011), *Penicillium*, *Rhodotorula* (Vogel et al., 2007), *Cladosporium*, *Mucor*, *Stachybotrys* (Bik et al., 2012; Gomes et al., 2008; González et al., 2000; Migahed, 2003), *Phialemonium* (Pong et al., 2014) and many others. *Trichophyton* and *Microsporium*, associated with skin and nail infections, also have been reported from beach sand (Sabino et al., 2011).

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

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

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

1.2 The Fungi

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

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

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

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

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

1.2.2 Allergenic moulds and endemic fungi

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

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

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

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

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

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

1.2.3 Dematiaceous fungi

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

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

1.2.4 Dermatophytes

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

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

The occurrence of fungi in soil can be influenced by non-biological factors such as soil temperature, humidity, rainfall, the chemical composition of the soil (including organic matter and pH) and sunlight irradiation (Coulibaly et al., 2016). Dermatophytes are no exception to this (Pontes et al., 2013). Dermatophytes are important microorganisms of the soil microbiota, with cosmopolitan species and others presenting a restricted geographic distribution (Pontes et al., 2013).

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

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

2. Materials and methods

2.1 Sampling sites

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

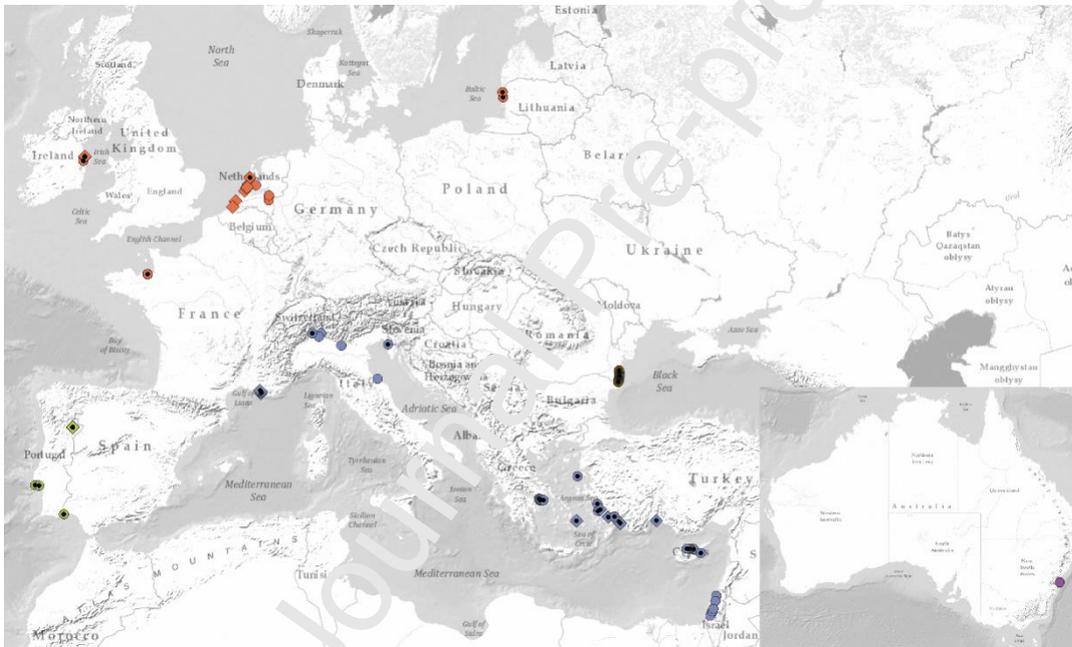
The following beaches were sampled for this project by Partners, as shown in Figure1 (Country codes follow ISO 3166-1 definition of AU=Australia (Sydney), IE=Ireland, FR=France, GR=Greece, IL=Israel, IT=Italy, LT=Lithuania, NL=Netherlands, PT=Portugal, RO=Romania, SL=Slovenia, RS=Serbia, TR=Turkey):

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

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

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

Figure 1: Geographical distribution of the sampling points using mapping with QGIS (Version 3.10.0-A Coruña). Circles correspond to urban beaches and diamonds to non-urban beaches. Dots within the shapes indicate water-sampling sites. Red=Northwest Europe, Green=Southwest Europe, Blue=Mediterranean, Brown=Black Sea and Purple=Sydney (Australia)



2.2 Analytical procedures

2.2.1 Sample preparation and incubation

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

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

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

2.2.2 Colony counting on plates after incubation.

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

2.2.3 Taxonomic identification of the colonies.

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

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

2.3 Statistical analyses

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

2.4 General Notes and categorisation

2.4.1 Samples

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

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

2.4.2 Parameters

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

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

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

2.4.3 Quantification

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

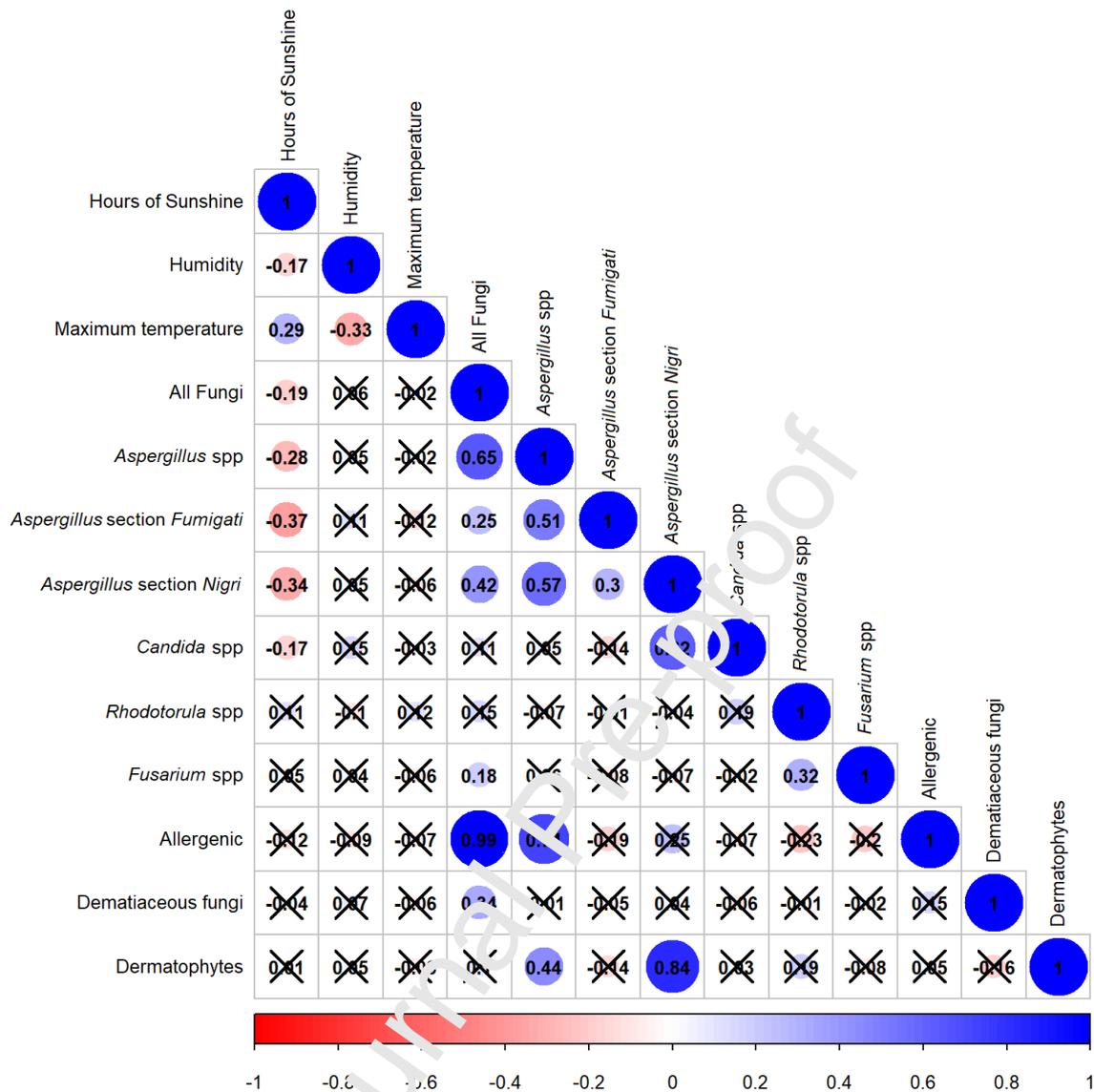
3. Results

3.1 Sand

3.1.1 Pearson’s correlation

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

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



3.1.2 Culturable mycobiota

The median number of fungal CFU/g in beach sand (of any kind, place, or period – All Fungi) was 89.2 CFU/g, with a range between 0.0 and 6400.0 CFU/g. By coastal or inland freshwater beaches, that 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, 6400.0) CFU/g for 42 inland freshwater beach sands ($p = 0.010$ - Table 1). Table 1 shows also that the distribution of fungi between them may be quite different. The parameters *C. albicans*, *Cryptococcus* spp., ‘Allergenic fungi’ and ‘Dematiaceous fungi’ appear with lower medians in coastal beach sands

than in freshwater beach sands, respectively 0.0 (0.0, 20.0) CFU/g to 29.2 (0.0, 50.0) CFU/g ($p=0.037$), 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, 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 suggests a difference in the typical composition of sand culturable mycobiota. Even 'Dermatophytes' shows a difference, these fungi are more present in coastal beach sands ((1.7 (0.0, 150.0) CFU/g) than in freshwater beach sands (0.0 (0.0, 166.7) CFU/g, $p=0.005$). The other parameters showed no statistical significance for this comparison. *A. section Flavi* (not shown in Table 1), *C. parapsilosis*, *C. tropicalis* and *C. dubliniensis* were reported only in coastal beaches.

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)	0.010	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)	0.016
<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, 2330.0)	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)	0.990	0.0 (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)	< 0.001	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)	0.037	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)	0.052	33.3 (0.0, 516.7)	1.7 (0.0, 126.7)	0.017
<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)	0.013	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)	< 0.001	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)	0.006
Dematiaceous fungi	0.0 (0.0, 2545.0)	19.2 (0.0, 600.0)	< 0.001	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)	0.005	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

3.1.3 Sand composition

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

3.1.4 Geography

The grouping of countries into regions (Table 2) was statistically significant only for some parameters of sand with Sydney (Australia) showing the highest concentration of several parameters of all regions: All Fungi ($p<0.001$), *A. section N gri* (<0.001), *Candida* spp. ($p=0.027$), *Rhodotorula* spp. ($p<0.001$), *Cryptococcus* spp. (0.0 (91), 'Dematiaceous fungi' (<0.0011), *Fusarium* spp. (< 0.001) and the dependent parameters Yeasts ($p<0.001$). Nonetheless, somewhat surprisingly the hotter climates are not necessarily the most (myco-) populated ones at the beach.

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)	90.3 (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, 155.1)	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, 21.7)	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, 30.0)	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, 100.0)	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	

Note: N=Number of samples

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

3.1.5 Period of the year

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

3.1.6 Urban versus non-urban

Candida spp. and *C. dubliniensis* are more prone to non-urban environments, as shown in (Table 1), with respectively, 4.2 (0.0, 555.0) CFU/g compared to 0.0 (0.0, 250.0) CFU/g in urban environments ($p<0.001$) 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.

Rhodotorula spp., *Cryptococcus* spp., *C. albicans* and *C. parapsilosis* were only detected in urban beaches. The *Rhodotorula* spp. results were 0.0 (0.0, 666.7) CFU/g in non-urban beaches, compared to 0.0 (0.0, 1333.3) CFU/g in urban beaches ($p=0.082$), as medians. However, with means of 47.2

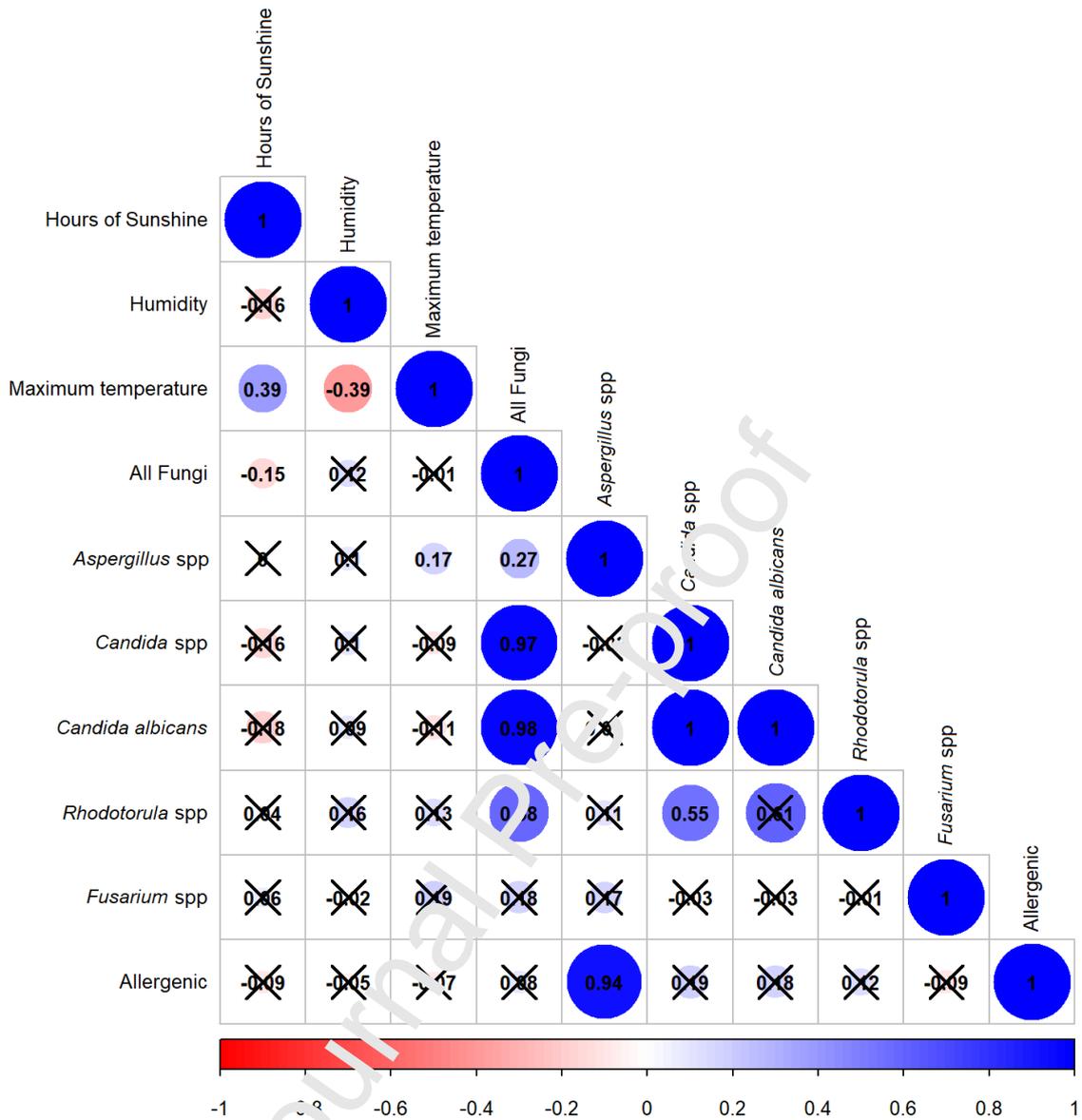
CFU/g for non-urban beaches and 76.6 CFU/g for urban beaches, the very high Standard Deviations (SD) take away any strength to the original behaviour expectation (SD=160.5CFU/g and 213.7CFU/g, respectively). 'Dematiaceous fungi', for which the median provides no relevant information or p-value either when comparing non-urban and urban beaches (0.0 (0.0, 600.0) CFU/g and 0.0 (0.0, 2545.0) CFU/g, respectively, $p=0.125$), the means and standard deviations are respectively 53.3 (133.3) and 27.2 (191.7), granting some expectation that maybe with more isolations 'Dematiaceous fungi' would possibly confirm a non-urban preference. Unlike *Fusarium* spp. which also has no statistically relevant difference (0.0 (0.0, 400.0) CFU/g for non-urban and 7.5 (0.0, 428.3) CFU/g for urban, but almost ($p=0.068$), that shows means of 28.7 (SD=79.2) and 47.8 (SD=98.1) respectively.

3.2 Water

3.2.1 Pearson's correlation

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

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



3.2.2 Culturable mycobiota

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

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

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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, 21.0)	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)	0.051	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,	0.0 (0.0, 98.3)	0.188	0.0 (0.0,	0.0 (0.0,	0.51	0.0 (0.0,	0.0 (0.0,	0.80

	1585.0)			1585.0)	250.0)	3	1585.0)	250.0)	9
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)	0.15 0	3.3 (0.0, 131.7)	1.7 (0.0, 13.3)	0.01 5
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)	0.87 8	0.0 (0.0, 33.3)	0.0 (0.0, 111.7)	0.00 6
Dermatophytes	0.0 (0.0, 1.7)	NA		0.0 (0.0, 1.7)	0.0 (0.0, 0.3)	0.66 1	0.0 (0.0, 1.7)	0.0 (0.0, 0.3)	0.45 0

The following parameters distribute themselves significantly between coastal and freshwaters in the following way, respectively: *C. albicans* - 0.0 (0.0, 170.0) CFU/ml to 3.3 (0.0, 8.3) CFU/ml, $p < 0.001$, 'Allergenic fungi' -1.7 (0.0, 13.3) CFU/ml to 5.8 (0.0, 131.7) CFU/ml, $p = 0.001$ and 'Dematiaceous 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. parapsilosis*, *C. tropicalis*, and *C. dubliniensis* and 'Dermatophytes' were only reported in coastal beaches (Table 3).

3.2.3 Geography

Regarding the parameters distributed by country and by region (Table 4), there are no noticeable regional differences in fungal parameters in bathing water, besides for *Aspergillus* spp., which show a p-value of 0.041 for the comparison between the Mediterranean, Northwest Europe and Southwest Europe regions only. The medians (and ranges) of this comparison are respectively 0.0 (0.0, 201.7) CFU/ml, 0 (0.0, 181.7) CFU/ml and 0.0 (0.0, 53.5) CFU/ml and mean values (and standard deviations) 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 adds statistical significance for 'Dematiaceous fungi' (0.0 (0.0, 111.7) CFU/ml for the Mediterranean, 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, with a $p < 0.0011$.

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, 1,911.7)	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)	0.0041
<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, 13)	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)	< 0.001
Dermatophytes	NA	NA	0.0 (0.0, 1.7)	NA	

Note: N=Number of samples

3.2.4 Period of the year

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

3.2.5 Urban versus non-urban

We can find differences in the distributions of fungi in water samples by urbanisation type in the parameters 'All Fungi' with 3.3 (0.0, 1591.7) CFU/ml for non-urban beaches and 0.0 (0.0, 310.0) CFU/ml for urban beaches ($p=0.005$); in *Aspergillus* spp. with 0.0 (0.0, 33.3) CFU/ml for non-urban beaches and 0.0 (0.0, 201.7) CFU/ml for urban beaches ($p=0.014$); and in *A. section Fumigati* with 0.0 (0.0, 20.0) CFU/ml for non-urban beaches and 0.0 (0.0, 90.0) CFU/ml for urban beaches ($p=0.008$).

4. Discussion

Environmental fungal exposure is recognized as being associated with a range of adverse health effects, including infectious or allergic diseases and toxic reactions. Efforts have been made in standardizing methods to assess indoor fungal contamination (Méheust et al., 2013). Only limited data on sand and water contamination are available, other than the ASTM D4249-83(2005) (ASTM D4249-83(2005), 2005). *Candida* Standard, withdrawn in 2013 due to its limited use by the industry. The major strengths of the Myco sands initiative are providing a large amount of original data and the standardization of methods between thirteen countries worldwide.

4.1 Sand

The results of this study, reporting a great diversity of fungi in the sand of bathing beaches, reinforce the findings of previous research (Abdallaoui et al., 2007; de Moura Sarquis and de Oliveira, 1996; Dunn and Baker, 1984; Echevarría, 2019; Efstratiou and Velegraki, 2010; Frenkel et al., 2020; Gomes et al., 2008; Kishimoto and Baker, 1969; Müller, 1973; Papadakis et al., 1997; Romão et al., 2017a; Romão et al., 2017b; Salleh et al., 2018; Tokura, 1984; Ulfig et al., 1997; Vezzulli et al., 2009; Yee et al., 2016). A variety of fungi have been identified in coastal and inland recreational beaches by other

research teams (Arvanitidou et al., 2002; Di Piazza et al., 2017; Efstratiou et al., 1998; Muntañolacvetković and Ristanović, 1980; Oliveira et al., 2011; Papadakis et al., 1997; Romão et al., 2017b; Rudenko et al., 2011; Sherry et al., 1979; Vezzulli et al., 2009). We identified over 300 taxa, which is, to the best of our knowledge, and this is the largest number of taxa ever reported from beach sand and water up to now.

During our study, we isolated a median which is lower than what Stevens *et al.* (Stevens et al., 2012) determined, with mean populations of 109.36 CFU/g in scarcely used coastal beaches, 140.49 CFU/g in averagely used beaches and 472.29 CFU/g in heavily used beaches. Dunn & Baker (Dunn and Baker, 1984) reported from 1 - 15,900 CFU/g fungi from beach sand of Hawaii. Much smaller numbers were observed by Echevarría (Echevarría, 2019) from the sand of beaches of Costa Rica (6 to 17 CFU/g), but they were isolating only filamentous fungi. Salvo & Fabiano (Salvo and Fabiano, 2007) isolated 0 to 11,470 CFU/g of yeasts and 55 to 1,778 CFU/g of filamentous fungi in the northwestern Italian beaches. Migahed (Migahed, 2003) in Egypt, reported that the total count of fungi in marine sand beaches ranges between 49.7 CFU/mg to 149.93 CFU/mg. It is not easy to compare and/or draw concrete conclusions concerning the abundance of fungi on beach sand, as this depends heavily on the overall pollution of the beach, the time of the year, environmental conditions such as temperature, UV radiation, precipitation events, the numbers of bathers, the grain size and the chemical nature of the sand, organic load, the presence of animals and birds. What is undeniably established by our work and that of other teams, is that the sand of bathing beaches is seeded with fungi.

Regarding the research published on the fungal load of sand in inland, freshwater beaches, we only identified one research article (Zatoń and Błaszczak, 2015) that isolated fungi in the sand of an urban lake beach in Poland, their numbers ranging between 1700 CFU/g and 2800 CFU/g. Considerably higher than the median abundance of all fungi observed in our project. In this area, we isolated significantly more fungi (as All Fungi numbers) from freshwater beaches than from marine beaches ($p=0.010$). This could be attributed to the more favourable environmental conditions (organic load,

lack of salinity, vegetation nearby). Additionally, freshwater beaches harboured in the sand significantly more *C. albicans* ($p=0.037$) as well as 'Allergenic fungi' ($p<0.001$), 'Dematiaceous fungi' ($p<0.001$) and 'Dermatophytes' ($p=0.005$). The inland beaches examined in this project were few compared to the marine beaches (little above 13%). This result may hence be seen as an indication of a trend, but more investigation would be needed to give a clearer picture.

Our findings on the influence of the presence of bathers on overall fungal numbers/diversity in sand suggest more isolates in urban beaches. However, the difference between urban and non-urban was not statistically significant ($p=0.344$). *C. albicans*, *C. parapsilosis* and *Cryptococcus* spp. were only detected in the sand of urban beaches. Our results reinforce the findings of several research groups claiming that the presence of humans influences the numbers and species of fungi on beach sand (Bergen and Wagner-Merner, 1977; Elmanama et al., 2003; Gomes et al., 2008; Kishimoto and Baker, 1969; Londono et al., 2018; Muntañolacvetković and Pisanović, 1980; Müller, 1973; Papadakis et al., 1997; Rudenko et al., 2011; Salvo and Fabian, 2007; Stevens et al., 2012; Vezzulli et al., 2009; Vogel et al., 2007). This can be attributed to the fact that many isolates are opportunistic human pathogens, easily shed into the environment. Statistically significant differences between urban and non-urban beaches were observed in the concentrations of *Candida* spp. ($p<0.001$) and *C. dubliniensis* ($p=0.052$), which appeared in higher concentrations in non-urban beach sand. This may be explained by *C. dubliniensis* having been reported to be associated with non-urban wild-life (Nunn et al., 2007).

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

Rhodotorula spp. and *Cryptococcus* spp. were expected to show a statistically significant presence in urban compared to non-urban beaches, under the premise that urban beaches may sustain some

level of pollution directly deriving from the urban run-off, pigeons and fossil energy hydrocarbons. Yet, only *Cryptococcus* spp. displayed that behaviour, to the extreme of not even having been detected in non-urban beaches.

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

We recorded statistically significant higher counts of All fungi in the Fall/Winter period, compared to the samples from the same sampling spots in the Spring/Summer period ($p=0.016$). Significant was the differences for *C. dubliniensis* ($p=0.017$) and 'Allergenic fungi' ($p=0.006$) also. *Aspergillus* spp., *Cryptococcus* spp., 'Dematiaceous fungi' and 'Dermatophytes' followed this trend, although not statistically significant. Previously published research results support our findings, as it has been reported that weather considerations can be important for the fungal load of sand beach. Sarquis & Oliveira (de Moura Sarquis and de Oliveira, 1996) and Assami Doi *et al.* (Doi *et al.*, 2018) reported that in the sands of Brazilian beaches the largest number of filamentous fungi was isolated during the winter and the smallest number the summer. The contradictory results of Stevens *et al.* (Stevens *et al.*, 2012) and Salvo & Fabiano (Salvo and Fabiano, 2007), who recorded larger numbers of yeasts in May/June/July than in September can be attributed to the fact that they did not sample during the winter. Dunn & Baker (Dunn and Baker, 1984) reported from Hawaii that in sands of comparatively high temperature (51°C) the numbers of isolated fungi were significantly fewer, than at beaches with a sand temperature of $30\text{-}35^{\circ}\text{C}$. Cases, where fungal populations peaked outside the bathing period have been attributed to pollution events, like a rupture of a sewage pipe (Salvo and Fabiano, 2007). In fact, that was also visible during our study with one sampling site in the Southwest region of

Europe at the end of 2018, which suffered a rupture in the sewage piping facilities, resulting in the high fungal presence (September 2018) in the sand (200 CFU/g) and in the water (65 CFU/ml). The water cleared promptly upon reparation of the facilities but the sand remained contaminated for at least 3 months (105 CFU/ml and 3.3 CFU/ml in March 2019), compared to 55 CFU/g and 3.3 CFU/ml in July 2019).

Our results are explained by the fact that although fungi can survive a wide range of environmental conditions e.g. light, temperature and salinity (Anderson, 1979), some of these parameters can eventually become detrimental to survival for microorganisms on sand particles. Anderson (Anderson, 1979) reported that elevated seawater temperatures, as high as 35°C are considered stress conditions for the fungi and inhibit the growth of *C. albicans*, *T. mentagrophytes* and *T. cutaneum*. Only *Microsporium gypseum* responded positively to increased temperature. The sand habitat is severely influenced by environmental changes, particularly temperature and UV light exposure. Sand particles exposed to the sun can become very hot. When the ambient temperature is 32°C, the sand temperature can be over 49°C (Cohen, 2019). Furthermore, exposure of microorganisms to Ultra-violet radiation (UV) causes denaturation of proteins and damage to their genetic material. High temperature of the sand in the summer can inhibit the growth or destroy fungi, and this combined with prolonged UV exposure in the long summer days can account for our findings.

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

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

noted that although the beaches they examined shared several species, each beach had species unique to it.

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

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

4.2 Water

The results of the water analysis indicate an interest in determining fungi in water, especially in freshwater, given the opportunistic and almost exclusive human faecal natures of *C. albicans* and the intimate contact with bathers, as mentioned in the introduction. Under this premise, faecal indicator bacteria (FIB) currently used in bathing water quality regulation - *E. coli* and *enterococci* ((EU), 2006) may not be used in this study, which focuses on fungi only, but our results show potential human

faecal pollution in 45 of the 302 coastal bathing waters (15%) and 4 of the 14 bathing freshwaters (28.6%).

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

Whilst several research projects have dealt qualitatively with the issue of fungi in recreational waters, publishing on the diversity and the taxa detected, little was published on the abundance of isolated fungi. We isolated significantly more fungi (measured as 'All fungi') from freshwater than from coastal waters ($p < 0.001$). This could be attributed (as in the case of sand) to environmental conditions being more favourable to fungal survival and growth in freshwater bodies than the sea (organic nutrients, lack of salinity, vegetation). The freshwaters of the inland beaches harboured significantly more *C. albicans* ($p < 0.001$), 'Allergenic fungi' ($p = 0.001$) and 'Dematiaceous fungi' ($p < 0.001$). More specifically we detected from 0 to 1592 CFU/ml of All fungi from coastal waters (mean 0 CFU/ml). Velegraki-Abel *et al.* (Velegraki-Abel et al., 1987) isolated 30-1020 CFU/50ml yeasts from marine beach water, Aulicino *et al.* (Aulicino et al., 2001) from $1 - 10^3/100\text{ml}$ yeasts. From inland beach waters, we isolated from 0 to 310 CFU/ml of All fungi (mean 6.7 CFU/ml). Similar findings were published by Wójcik *et al.* (Wójcik et al., 2003) who isolated from 8 to 32800 CFU/100ml yeasts from reservoir water used for recreational bathing. Goralska *et al.* (Góralaska et al., 2020) detected an average of 40 to 460 CFU/100ml in recreational freshwater ponds in Poland.

The presence of bathers appears to affect the abundance of fungi isolated from beach water. Urban beaches have significantly more fungi (measured as All fungi) than non-urban beach water ($p = 0.005$).

Aspergillus spp. and *Aspergillus* section *Fumigati* are the two fungal groups that show significantly higher numbers in urban beaches ($p=0.014$ and $p=0.008$ respectively). Similar results have been reported for yeasts. Papadakis *et al.* (Papadakis *et al.*, 1997) claim that yeasts of human origin correlated with the numbers of swimmers in seawater. Velegraki-Abel *et al.* (Velegraki-Abel *et al.*, 1987), also investigating in Greek coastal bathing waters, reported that yeast counts increased during the summer. Sherry *et al.* (Sherry *et al.*, 1979) published that maximum numbers of *C. albicans* were observed in association with peak bather loads at the beaches of a lake in Canada.

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

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

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

4.3 GENERAL COMMENTS TO BOTH WATER AND SAND

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

Valdes-Collazo *et al.* (Valdes-Collazo et al., 1987) found that *C. albicans* survives well in freshwater and marine water, so bathing in its presence necessarily represents an exposure setting to this organism where intimate contact with the bather's skin and mucosae take place. Conversely, this species does not respond well to dehydration, which renders it to be a possible indicator of recent human faecal pollution in the sand and in water; its decay is similar to that of *E. coli*, as noted by Kashbur *et al.* (Kashbur et al., 1980).

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

The prediction of pathogen risks is essential to beach management. The increase of chances of an introduction of fungal pathogens into the population should be avoided, because of the increased numbers of immunocompromised people, the advances in chemotherapeutic treatments that allow patients to move around and visit recreational spots, as well as the ageing of the human population. Identifying the most important routes of fungal transmission in the sand would allow beach management officials to improve services and reduce risks of exposure to pathogenic fungi. Little research has been published on within-one-beach differences in fungal populations (Brandão et al., 2020a; Velegraki et al., 2012) providing evidence that sand around showers exhibits the highest

numbers of keratinophilic isolates, followed by sand from children's playground areas and from sports activities areas. Should such findings be supported by further research it would be possible for beach managers to improve the management of shower run-offs or the treatment of shower effluents or relocate showers in order to avoid spread/unnecessary contact.

5. Conclusions

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

Fungi are nature's organic matter recycling machines. Different species may thrive mainly with specific substrates, rendering them good indicators for specific situations. The presence of *Fusarium* species in the sand, for example, may indicate remains of vegetable debris, as these species are plant pathogens and colonisers. Another group highly associated with densely vegetated areas are the Melanised fungi. These include species that cause deep and often lethal infections, as well as allergies. A list of the genera found in this study can be found in the supplementary material.

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

It is the opinion of the authors that the monitoring of fungi in beach sand and water is relevant, particularly for the most susceptible beach users. The authors of this study also consider that at least

C. albicans and dermatophytes should also be monitored as additional health-oriented parameters (besides total fungal colony count). Other possible fungal parameters, species or genera should be considered on a need-to-do basis, according to persistent pollution or specific health endemic requirements.

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

Highlights

- Fungi are missing from water and sand health protection regulatory parameters.
- Both sand and water should be monitored for fungi
- The median value of 89 CFU/g of all fungi may serve as a reference for sand regulation
- *Candida albicans*, dermatophytes, endemic fungi and other fungi should be considered
- Fungal analysis of water needs more data before reference values can be established

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Declaration of interests

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

Author contributions (CRediT author statement)

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