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Departamento de Biologia

Beach sand microbiology: geo-climatic contextualization and public health implications

"Documento definitivo"

Doutoramento em Biologia e Ecologia das Alterações Globais

Biologia Ambiental e Saúde

João Carlos Simões Brandão

Tese orientada por:

Doutora Raquel Filipa Pinheiro Sabino (INSA)

Professora Doutora Maria Teresa Rebelo (FCUL)

Documento especialmente elaborado para a obtenção do grau de doutor

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Nota prévia

A presente tese apresenta artigos científicos já publicados, submetidos ou em preparação para publicação (capítulos 2 a 8), de acordo com o previsto no nº 2 do artigo 25º do Regulamento de Estudos de Pós-Graduação da Universidade de Lisboa, publicado no Diário da República, 2.ª série — N.º 57 — 23 de Março de 2015. Uma vez que estes trabalhos foram realizados em colaboração, o candidato esclarece que participou integralmente na conceção dos trabalhos, obtenção dos dados, análise e discussão dos resultados, bem como na redação dos manuscritos.

Lisboa, Abril de 2021

João Carlos Simões Brandão

I ola oe, I ola makou nei

Hawaiian proverb meaning ‘if you thrive, we thrive’ (said when things are planted – also ideas).

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Dedicated to my parents, Fernanda, and Fernando Brandão

Resumo Alargado

Uma praia cuja água balnear é de boa qualidade significa que é possível tomar banho com uma probabilidade negligenciável de adoecer por exposição a agentes patogénicos propagados na água. Esta questão, no entanto, não fica completa só com análises da água, já que a maior parte do tempo de estadia na praia não é na água. A areia tornou-se objeto de estudo há algumas décadas, mas por alguma razão indefinida, a implementação de parâmetros de qualidade desta matriz não ocorreu até recentemente. Em 2017, a Argentina deu o primeiro passo e acrescentou a inspeção de areia ao regulamento nacional de águas balneares e em 2018, a Lituânia acrescentou a pesquisa de helmintas em areias. A necessidade de monitorização da areia foi validada em 2003 com a publicação das *Guidelines for safe recreational water environments* pela Organização Mundial da Saúde. Desde então, as publicações continuaram a surgir num ritmo de crescimento lento, mas sem recomendações claras de parâmetros de monitorização; a água continuou a ser o foco exclusivo das regulamentações nacionais em todo o mundo durante quase quinze anos. Em 2012, foi publicado o primeiro relato de possíveis efeitos na saúde causados por contacto com areia num artigo de um estudo epidemiológico realizado nos Estados Unidos da América. Em 2015, foi publicada uma carta aberta recomendando metodologias de monitorização da areia, explicando os motivos pelos quais esta deve ser monitorizada. Esta publicação surgiu como resultado de um encontro internacional e cobre temas como a exposição direta a microrganismos, que poderá eventualmente acontecer na praia, independentemente do nível de contacto, e a subida dos valores dos parâmetros de qualidade da água por escorrências. Os excrementos de pássaros depositados na areia são um conhecido contributo para a subida dos níveis de bactérias indicadoras de contaminação fecal na areia e, consequentemente, nas águas adjacentes. Esta situação pode conduzir à interdição da praia devido a valores elevados na água, de acordo com regulamentação. Pelo exposto, a qualidade microbiológica da areia também precisa ser regulamentada.

Neste contexto, esta tese foi elaborada para preencher lacunas no conhecimento e produzir uma recomendação sustentada sobre métodos, parâmetros e necessidades a colmatar no futuro. O primeiro capítulo da tese é constituído por uma introdução que resulta da revisão de uma seleção de *publicações-chave* em qualidade de areias e águas balneares. Seguem-se sete capítulos técnicos que resultam do trabalho realizado durante os quatro anos da formação. São identificadas as falhas no conhecimento corrente sobre o tema de estudo e explicada a relevância de uma miríade de variáveis que influenciam todo o cenário balnear.

O segundo capítulo reflete um estudo de grupo realizado com o objetivo de avaliar a influência das alterações climáticas no paradigma areia-indicadores-agentes patogénicos-saúde humana. Foi possível estimar alguns impactos de interesse, e enfatiza-se que o painel de parâmetros atualmente utlizados terá provavelmente de ser expandido para refletir agentes patogénicos não endémicos (e.g. *Vibrio colerae* e *V. parahaemolyticus* atualmente em expansão para algumas águas mais a norte dos seus habitats naturais).

O terceiro capítulo apresenta o trabalho publicado num capítulo de uma enciclopédia de saúde ambiental que pretendeu colmatar algumas abordagens não exploradas como: amostragem de areia e parâmetros a analisar, resistência a antimicrobianos e avaliação de risco neste contexto. Relativamente à amostragem é discutida a sua representatividade da praia de onde provém, devendo ser feito um esforço para coletar tantas frações quanto possível, de forma a constituir uma amostra composta cuja representatividade apresente alguma robustez. O pior cenário previsível deve ser dominante na amostragem, já que o objetivo das análises que se seguem é proteger tanto quanto possível a saúde dos visitantes da praia. As zonas balneares estão atualmente a ser alvo de pesquisa de genes de resistência a antimicrobianos que se encontram cada vez com mais frequência em águas balneares, normalmente atribuídas a escorrências e poluição difusa. A areia da praia faz assim parte do processo de propagação de genes de resistência.

O capítulo quatro apresenta uma contribuição para um módulo de referência em ciências da vida sobre fungos em ambientes recreativos, que inclui preocupações sobre exposição em contextos balneares de praias, águas de piscina, exposição em contextos de campismo e jardinagem, caixas de areia e exploração de grutas, considerando ainda avaliação de risco e alterações climáticas nesses contextos recreativos. Fungos negros são um grupo de fungos muito frequente em piscinas por serem resistentes à cloração, sendo alguns produtores de micotoxinas. Também sobre jardinagem e campismo se referem as micotoxinas produzidas por *Wallemia* spp. (wallimidiona, walleminona e walleminol A) e infecções por *Sporothrix schenckii*. Aborda-se o risco de exposição a *Histoplasma* spp. em cavernas com morcegos, em exploração e espeleologia. Relativamente a areias são exploradas as areias de caixas para as crianças brincarem e as areias de praias em combinação com dose fúngica de exposição e alergias a espécies alergénicas. Por último, considerando alterações climáticas, discute-se a expansão para norte da espécie *Cryptococcus deuterogatii* e de infeções causadas por fungos da Classe Mucormicetes, resultantes de inoculação direta com fragmentos de madeira

projetados pelo ar durante furacões e o estudo de avaliação de risco em exposição a fungos em contextos ambientais.

O capítulo cinco aprofunda o estudo de avaliação de risco de exposição a fungos, área que não tem feito parte dos objetos de estudo dos profissionais dessa área temática relativa a fungos endémicos, já que para os oportunistas não são conhecidas doses infeciosas que são a base dos referidos estudos. Sem esses estudos, que resultam de estudos epidemiológicos de exposição e dependentes da articulação entre a comunidade médica e epidemiologistas, não é possível explorar esta abordagem para os fungos que é urgente investigar.

O capítulo seis apresenta o resultado de um projeto exploratório de avaliação da presença de fungos em águas e areias balneares de grande envergadura geográfica, com o objetivo de avaliar a flora fúngica "normal" existente em praias de todos os tipos e com climas diversificados, com a exceção de climas tropicais e glaciares. As bacias hidrográficas integradas no estudo vão desde a costa Atlântica ao Mediterrâneo oriental, incluindo os lagos do norte de Itália e os mares Adriático, Báltico, Negro, e a bacia de Sydney, na Austrália. Participam neste projeto treze países, dos quais foram analisadas areias de noventa e uma praias, tendo sido a água de sessenta e sete destas também testada. Determinou-se um valor mediano geral de 89 Unidades Formadoras de Colónias (UFC) por grama de areia; valor este que pode servir de guia orientador em estudos de praias sem histórico de análises realizadas, e que foi integrado nas recomendações das novas *guidelines* para águas balneares da Organização Mundial de Saúde. Os géneros fúngicos mais frequentemente detetados em águas e areias foram *Aspergillus* spp., *Candida* spp., *Fusarium* spp. and *Cryptococcus* spp. Encontraram-se associações com as variáveis analisadas: praia costeira ou interior, praia urbana ou não-urbana, época do ano, proximidade geográfica e tipo de sedimento. Revelou-se maior carga fúngica nas praias costeiras do que nas de interior. No período do outono e inverno verificou-se a existência de areias mais densamente contaminadas por fungos, maioritariamente de origem ambiental. Não se observou influência positiva do sol nos fungos demaceáceos, que usam radiação como fonte de energia.

O capítulo sete apresenta um estudo de um surto de dermatite maculo-eritematosa com prurido em 30 crianças durante um dia na praia. Algumas destas crianças não entraram na água pelo que se confinou a origem da possível infeção à areia; a sintomatologia sugeria contacto direto com o agente irritante por só se verificar em zonas do corpo não cobertas. Iniciou-se a investigação para encontrar o agente responsável do surto, analisando-se bactérias indicadoras

de contaminação fecal, fungos, cloro livre, nitratos, nitritos e amónia e compostos voláteis aromáticos. O valor alto das bactérias indicadoras de contaminação fecal, levou a equipa local a procurar possíveis fontes de contaminação. Foi encontrada uma caixa de derivação de esgoto deteriorada e que escorreu águas residuais, fortemente carregadas de lixívia usada na limpeza de início de época balnear no bar contíguo à praia. O agente irritante foi confirmado por espectrometria de massa cromatográfica onde se detetou um pico compatível com hipoclorito de sódio. A praia foi reaberta após reparação da caixa e substituição da areia contaminada até meio metro de profundidade.

Finalmente, os capítulos oito e nove abordam regulação e recomendações de gestão de praias. O capítulo oito incide nas novas *guidelines* da Organização Mundial de Saúde, onde se abordam vários pontos de interesse em gestão de microbiologia de areias de praias e se faz a recomendação para a análise de enterococos, usando como limite de referência 60 UFC/g, que corresponde a 5% de risco de doença por exposição em águas balneares. É ainda recomendada a monitorização de fungos usando o valor determinado pelo estudo do capítulo seis como valor guia para gestão de praias sem historial analítico.

O capítulo nove apresenta ainda uma recomendação global de parâmetros, métodos e valores limites a usar na gestão microbiológica de areias de praia, concluindo com a identificação de pontos de interesse para estudos futuros de forma a contribuir para o desenvolvimento do conhecimento nesta área.

Palavras-chave

Microbiologia de areias, Água balnear, Fungos, Dermatófitos, FIB

Abstract

Good recreational water quality at a beach means that it is possible to bathe with a negligible probability of becoming ill, from exposure to waterborne pathogens. However, this does not tell the whole story since most of the time spent at the beach is not in the water. Sand became a subject of study a few decades ago, but for some reason, the implementation of quality standards never took place until recently. In 2017 Argentina took the first step and added sand inspection to the national bathing water regulation. In 2018, Lithuania added helminths in sand to its own regulation. Yet, the validation of the need to monitor sand happened in 2003, with the publication of the Guidelines for safe recreational water environments by the World Health Organisation. Since then, publications continued to emerge at a slowly on-growing pace but without clear recommendations of monitoring parameters and levels, water continued to be the focus of national regulations everywhere for almost fifteen years. In 2012, a paper was published with the first report of health effects of unmonitored sand, during an epidemiological study performed in the United States of America. In 2015, a broad white paper was published recommending methods and stating reasons for monitoring sand. It was the result of an international meeting and covers direct exposure by being at the beach, regardless of the level of contact, and water quality parameters levels rise due to run off. Bird droppings on sand are a known contribution to faecal indicator bacteria levels in sand and consequently in adjacent waters. Beaches can get closed due to their excessive presence, according to regulation. Sand needs therefore to be regulated too. This thesis was designed to fill gaps in the knowledge and produce a sustained recommendation on methods, parameters and needs-to-do in the future.

Keywords

Sand microbiology, Bathing water, Fungi, Dermatophytes, FIB

Table of contents

Chapter 1

Introduction

1.1 General introduction

Sand is composed of minerals and water sustaining complete micro-ecosystems that comprise all kinds of biological forms. Bacteria and fungi interlace in complex biofilms attached to the surfaces of the grains of sand, thriving on the nutrients available and sunshine as the energy source (Weiskerger *et al.* 2019).

A day at the beach is spent mainly in the sand area where people lie down sunbathing and where children play. The wind displaces loose grains of sand, some of which end up deposited on our skin and hairs, forced into our ears, nose and mouth. At the end of that day, we go home, inevitably taking some along with us. Yet, despite the amount of time spent on sand being higher than what is spent in water, the latter has well established microbial exposure/quality safety standards, but sand is lagging (Weiskerger *et al.* 2019; WHO, 2003).

The first paper published on beach sand contaminants was in 1960 by Schönfeld, Rieth and Thianprasit, and it described a study aiming to detect the presence of dermatophytes in a Baltic Sea resort. The study yielded only the geophilic dermatophyte *Arthroderma insingulare* (formally *Trichophyton terrestre*), and in the superficial layers of supratidal sand (Schönfeld *et al.* 1960). In 1973, however, Gertrud Müller confirmed the presence of anthropophilic dermatophytes in sands of a wider study. For this research, Müller's team sampled Estoril, near Lisboa, the Adriatic Sea, at Gabicce Mare and at Grömitz, on the German Baltic coast. The team searched for *Epidermophyton floccosum*, an anthropophilic species, for three years, and sampled twice a week (in the same places). They also aimed at finding beach usage associated variations of this fungal species (Müller, 1973). The study revealed a clear surge of the presence of *Epidermophyton floccosum* in sands touched by human bare feet. It failed to isolate other species, common in other kinds of soil, showers, and pools such as *Trichophyton interdigitale (*formerly *T. mentagrophytes var. interdigitale*) or for *Nannizzia gypsea* (formally *Microsporum gypseum*). The authors pointed out, in their report, that the inability to isolate these common agents of dermatophytosis should not rule out their presence in stepped-upon beach sand.

Maria Colon Valiente, in 1990, hypothesised that the absence of dermatophytes in beach sand was probably due to the low nutrient environment or most likely to the high temperatures that the sand reaches (Valiente 1990). These observations, however, may be challenged by Sousa (1990), who reports dermatophytes in 11 out of 24 beaches studied of coastal areas around Lisbon. Brandão *et al*. (2002) sampled all the regional coasts of

Continental Portugal every 2 months, for 13 months, and generated 210 samples of sand from both the supra-tidal and the vadose zones. In this study, a higher density of beach users also correlated with higher levels of dermatophytes, thus, during the summer months. Recently, *E. floccosum* seems to be decreasing its prevalence in clinical specimens worldwide, indicating that the probability of isolating it from beach sand may completely disappear (Zhan and Liu, 2017). Other dermatophytes, and even other microbial life forms, may alter their patterns of existence due to clinical intervention and climatic alterations. For example, as desertification takes place in some areas of the globe, human communities are expected to settle more near the coastlines, where temperatures are more agreeable (Weiskerger *et al.* 2019).

The rationale that we spend more time on the sand at a beach than in the water was clearly expressed by the World Health Organisation in 2003, with chapter 6 of the guidelines for safe recreational water environments (WHO, 2003). Between 1969 and 2003, many publications addressed sand in its multiple microbial fronts: Bacterial, Mycological, Parasitological and Viral. Yet, it was not until 2012 that proof of the relevance of sand exposure to the health of beach users arose, with the studies of C. Heany's team (Heany *et al.* 2012). Wondering if indeed sand could influence the health of beach users, the team analysed 144 wet sand samples and 4999 interviews comparing contact with sand could result in oral exposure to bacteria present in sand, with those who did not report any sand contact of that kind. *Enterococcus* spp. in sand was positively associated with gastrointestinal (GI) illness, among those digging in the sand or buried in sand compared with those who did not. The authors looked at these bacteria, due to being a Faecal Indicator Organism (FIO) or Faecal Indicator Bacteria (FIB) and known to correlate with human ailments after bathing in recreational waters, as explained in chapter 4 of the WHO's guidelines currently in place (WHO, 2003).

There are other organisms, which possibly indicate faecal contamination of bathing waters, such as *Cryptosporidium*, *Clostridium perfringens*, and *Bacteroides* spp. The latter are used mainly to track the biological source of faecal pollution (Fujioka *et al.* 2015). This paper was based on a live discussion about the recreational water quality criteria review/update of 2012 of the USA. One of the points discussed is how the current FIB may in the future need to expand to other organisms in water and to sand monitoring. Hawai'i has used *C. perfringens* as a FIB for decades with particularly good results. The rationale behind this is that *C. perfringens* performs better as a faecal indicator in tropical water temperatures. Considering the global warming situation, alternative indicators are expected to be employed to mitigate

exposure to waterborne pathogens of faecal origin (Fujioka *et al.* 2015; Teixeira *et al*. 2020; Weiskerger *et al.* 2019).

One of the current approaches recommended in 2004 by the WHO for drinking water is the establishment of water safety plans (WSP), to preventively minimize threats to water supply (systematically assess and manage risks) (WHO, 2004). This recommendation covers all possible fronts, including sediment. The European Commission readily adopted this perspective for the most recent Bathing Water Directive 2006/7/EC (EU, EEU, 2006). In the WHO 2020 review of the guidelines for safe recreational water environments, WSP are recommended also for recreational waters (WHO, *in press*). This emphasises the need to consider nearshore as a possible source of water pollution, as well as an established exposure route to pathogens and opportunistic organisms.

1.2 Types of sand and artificial beaches

Beach is "*the zone of unconsolidated material that extends landward from the low water line to the place where there is a marked change in material or physiographic form, or to the line of permanent vegetation (usually the effective limit of storm waves). The seaward limit of a beach – unless otherwise specified – is the mean low water line*" (Micallef and Williams, 2009). Exposed sandy beaches are physically dynamic habitats, inhabited by specialised biotic assemblages that are structured mainly by physical forces (Defeo *et al.* 2009). In 2016, Abreu and collaborators published a paper addressing sand grain size and composition crosschecked with microbial flora (Abreu *et al.* 2016). In this study, the statistical analysis showed that the sand granulometry and chemical composition did not influence the microbial concentration. In the same study, a comparison was made between natural and artificial beaches, revealing that in this case there is a difference - manmade structures built to maintain sand in place of artificially built beaches impede the natural wave activity and thus limit wash-off of microorganisms from sand, resulting in their higher concentrations in the supratidal area. This is the area mainly used for sunbathing and relaxing.

Beach nourishment is a common practice for reclaiming coastline, lost to wave activity and natural erosion, and to build artificial beaches (Bitan, *et al.* 2020; Hernandez *et al*. 2014). The source of the nourishing sand may be of relevance, as the nourishing sand will carry its own native microbial flora to the new location (Solo-Gabriele *et al.* 2016). When the artificial beach *Praia da Calheta* was built and nourished with sand originating in the Saharan desert, Morocco, the first batch of sand brought live scorpions along with it. Consequently, the project had to be

halted and redesigned (Outro Planeta, 2004). This event was never published in the scientific literature. Nourishing sand should thus originate as close as possible to its final location, in order not to carry along local life from the source of the sediment. The sand used to nourish a beach has been calculated to be, ideally, grain-sized 1.5 to 2.0 times the original sand, for the durability of the action (Bitan *et al.* 2020).

1.3 Swash zone vs supratidal zone

The sand area of a beach is divided into several sections. In this text, the focus shall be on the vadose or swash zone (the intertidal area limited above by the high tide reach) and on the supratidal zone (beyond wave and high tide reach). The latter tends to stay dry unless a weather event wets it by precipitation and run-off from the backshore.

Backshore run-off is a well-known means of sand contamination, as demonstrated by an episode of a destructive tropical storm hitting the island of Madeira in 2010 (Abreu *et al. 2016*). The storm destroyed facilities of all kinds, including sanitary infrastructures. Due to the heavy rain, land- and mudslides rushed down to the lower lands (coastal line). The faecal indicator bacteria (FIB) surge at the beach sand could be detected during approximately three months (Romão *et al.* 2017).

Conversely, the swash zone is extremely influenced by the waves and tides (Weiskerger *et al.* 2019). In 2011, a meeting in Michigan City planned a publication to review and describe the fate of the sand micropsammon (Bacteria, Fungi, Parasites and Viruses). In this publication, the authors describe how sand microbial communities include "*autochthonous species/ phylotypes indigenous to the environment. Allochthonous microbes, including fecal indicator bacteria (FIB) and waterborne pathogens, are deposited via waves, runoff, air, or animals*" (Whitman *et al.* 2014). The publication also addresses the naturalisation of allochthonous micropsammon, introduced by water, air, run-off and by animals. FIB are considered allochthonous to beach sand.

1.4 Microbial parameters

Several publications identify potential microbial culture parameters for sand monitoring, namely Abdelzaher *et al*. (2010), Sabino *et al.* (2014), Sato *et al*. (2005) and WHO (2003). Others contemplate meta-genomics, raising possible alternative parameters, based on nucleic acid analyses (Alwakeel, 2017, Mohiuddin *et al.* 2017; Romão *et al.* 2017; Taylor and Kurtz, 2020). However, until there is a regulatory document defining sand quality parameters, the choice for monitoring parameters is likely to fall on a combination of culturable FIO, to indicate

pathogens and opportunists associated with wastewaters, and others that do not relate to faecal pollution (like most fungi). NGS might, however, be an interesting screening tool to assess variations in the microbiota as shown in Romão *et al.* (2017) and in Taylor & Kurtz (2020). In this study, the authors looked at three beaches along the Grand Strand of South Carolina, United States of America (USA). The authors sequenced the V4 region of the 16S rRNA gene, to ascertain relationships between diversity and temporal or local factors. Gammaproteobacteria, Planctomycetes, Acidobacteria, and Actinobacteria were the dominating bacterial populations of these beaches. The communities were similar in overall composition and diversity but changed the levels of community structure over time.

Brandão *et al*. (2002) describe how the swash zone interconnects water and sand of the supratidal area of a beach. The authors conclude that water is monitored by default, due to regulation, so sand needs only to be monitored in the supratidal area, as the swash zone will not generate particularly useful information. The following three bullets describe the taxa of interest for sand monitoring. Chapters two to eight will detail further and contextualise within climate change scenarios and address four other biological groups of interest poorly represented in the scientific literature – viruses, helminths, and insects.

1.4.1 Faecal Indicator Bacteria

Faecal pollution remains one of the biggest problems with recreational waters and beach sand. Part of the globe has taken action to eliminate untreated sewage falling out into water bodies. But, in other parts, faecal contamination of bathing and even sources of drinking water is a serious problem. One of the most noticeable episodes took place in 2010 in Haiti, due to the destruction caused by an earthquake of 7.0 magnitude. This event killed an estimated 230,000 people and injured 300,000. Haiti had already a rather low coverage of sanitation but after the earthquake, only 10% of the rural population and 24% of the urban population had access to improved sanitation (WHO/UNICEF, 2012). The low rate of access to drinking water and sanitation led to an outbreak of cholera, which resulted in 658,563 reported cases of cholera and 8,111 deaths as of June 2, 2013 (Gelting *et al.* 2013).

The WHO guidelines for safe recreational water environments (WHO, 2003) recommends the use of FIB to detect faecal pollution in recreational waters, and the forthcoming guidelines (WHO, *in press*), also in the sand, precisely to avoid waterborne diseases originating from human excreta. The actual pathogens are not currently looked for, only the FIB. According to the literature, exposure to 40 CFU/ml of enterococci results in an illness probability of 1%, and

up to 200 CFU/ml results in up to 5% probability of illness. This means that this parameter in recreational waters indicates disease probability rather than any specific pathogen-associated illness. Fujioka *et al.* (2015), Weiskerger *et al*. (2019) and Teixeira *et al*. (2020) discuss how in the future we may have to look beyond FIB, to accommodate changes arising from climatic alterations. Looking into pathogens directly instead of just quantifying surrogates is not only increasingly easy but will also have to be implemented for pathogens that will challenge the current paradigm of recreational water quality criteria.

1.4.2 Other Bacteria

As described in WHO (2003), Sabino *et al.* (2014), and Weiskerger *et al*. (2019), controlling exposure to bacteria to protect human health needs to go beyond FIB, since the supratidal zone of a beach should not be greatly contaminated with faecal pollution. Instead, its main source of pollution should be skin shedding from animals, vegetable debris and other organic matter that may serve as food for wildlife (Brandão *et al.* 2002).

The following bacterial taxa have been considered of relevance for the protection of human health: *Staphylococcus* spp. (skin infections), *Vibrio* spp. (cholera and necrotizing fasciitis), *Clostridium perfringens* (food poisoning possible cause of bacteraemia), *Campylobacter jejuni* (gastroenteritis) and *Shigella* spp. (haemorrhagic diarrhoea), *Pseudomonas aeruginosa* (superficial and systemic infections) (Sabino *et al.* 2014; WHO 2003). This group of sand contaminants and climate change implications to some of these organisms is addressed further in WHO (*in press*) and in Weiskerger *et al.* (2019).

1.4.3 Fungi

Most fungi are opportunistic, which means that exposure is risky only for susceptible individuals. Yet, two groups of fungi are of great concern: dermatophytes and endemic fungi. Dermatophytes are keratinophilic fungi that cause dermatophytoses, superficial infections of the hair, nails, skin, and scalp. There are anthropophilic, geophilic and zoophilic dermatophytes and they are so classified according to the transmission route: if humans to humans, soil to humans or animals to humans, respectively (Hainer, 2003). The current knowledge of these fungi in sand suggests that anthropophilic dermatophytes may not withstand the harsh conditions of beach sand and thus die out rather quickly. This does not necessarily mean that there is no transmission at the beach. Shedding takes place naturally, especially from infected areas which become dry and scaly, and death due to radiation or high temperatures is not immediate. In fact, in a laboratory conditions recreation of beach sand from Hawaii, Anderson

(1979) tested the survival of *Trichosporon cutaneum (T. asahii), Candida albicans, Microsporum gypseum* and *Trichophyton mentagrophytes (*var. *mentagrophytes)* and all survived at least for one month in non-sterile sand inoculated with keratinized propagules. These data suggest that if other authors have difficulty isolating the more anthropophilic dermatophytes, the cause may be the dispersion of propagules and representativity of sampling, rather than the survival itself. Growth conditions and media may also be the cause of the inability to isolate specific fungi.

As for the endemic fungi *sensu stricto* (*Coccidiodes* spp., *Paracoccidiodes brasiliensis*, *Histoplasma* spp., *Blastomyces dermatitidis*), no known publications indicate their isolation from beach sand. However, their natural habitats suggest some degree probability of survival in beaches of endemic areas (inland, mainly). *Coccidiodes* spp. inhabits dry, desert-like territories of the USA, and of Argentina and the remaining are endemic to more humid habitats [Mississippi river valley (*Blastomyces* spp.), central and eastern states USA (*Histoplasma capsulatum*), *Histoplasma duboisii* (Subsaharan Africa) and South America (*Paracoccidioides brasiliensis*)] (Salzer *et al.* 2018). *Cryptococcus deuterogattii* is also endemic to the Pacific Northwest of the North American continent (Applen Clancey *et al.* 2019) but not considered dimorphic (real pathogens). There is yet to be consensus weather this species should stand on its own or be considered simply a genetic type of *Cryptococcus gatii* (type AFLP6/VGIIa).

Other fungi of interest for exposure in natural environments are *Mucorales*, allergenic fungi and dematiaceous fungi. *Mucorales* is the order that includes the fungi responsible for the invasive mucormycosis. This order has raised some concern due to the opportunistic ability to start an infection in immunocompetent individuals when inoculated deeply under the skin by piercing materials and because of its intrinsic resistance to Voriconazole. This has been well demonstrated by a cluster of cases of necrotizing cutaneous mucormycosis following a tornado in Joplin, Missouri, in 2011 (Neblett Fanfair *et al.* 2012). It is also a relevant group of fungi in cases of near-drowning, as described by Sympardi *et al*. (2019).

Considering that an allergy is host-dependent and that there are individual allergies to most fungi, for the purpose of this text, the definition of allergenic fungi shall be any fungus. There are, however, different types of allergies, respiratory and contact allergies. Respiratory allergies imply inhaling allergens and therefore mainly caused by air-borne-sporulating fungi. The extent to which fungal spores can travel air-borne has been clearly described by Kellogg and Griffin (2003), in an open report about the air-travel of fungi across all the Atlantic Ocean,

from Africa to North America. Fungal spores have this the ability to be airborne and surely to extend their presence to an entire beach.

Although the information on fungal inhalation leading to infections specifically from sand is yet unavailable, Buskirk *et al*. (2014) showed in a murine model that the dry exposure to 105 spores of *A. fumigatus* twice a week, triggers an inflammatory response in the lungs at 24 and 48 hours thereafter. Additionally, an IgG elevation is observed after 7 days, concomitant with spore germination. Tanaka *et al* (2015) found that cytokines release in immunocompetent individuals takes place about 19h post-inhalation. Cho *et al*. (2005) observed in fungal respiratory deposition models that particles of *Stachybotrys chartarum* may be deposited 230-250-fold higher than spores. These data suggest a delayed first response to the exposure to fungal allergens, but an existing one, nonetheless. Beach users might thus not even associate an allergic episode with a visit to the beach the day before. Lastly, exposure to volatile organic compounds (VOC) and produced by fungi, like 3-Methylfuran, may cause nonspecific symptoms, namely eyes, nose and throat irritations, headaches, and fatigue (Wålinder *et al.* 2005). Also, of some concern, especially in indoor setting are Mycotoxins, which the mentioned fungal taxa may produce causing several possible pathologies (Tola *et al.* 2016).

Dematiaceous or melanised fungi are taxa that produce melanin as a means to harness energy from radiation to use in biochemical paths. The most common ailments associated with melanised fungi are keratitis, cutaneous, subcutaneous, and respiratory tract infections. Exposure to sand combined with a traumatic event may originate an invasive fungal infection (phaeohyphomycosis). The severity depending on the extension of the trauma and immune response of the host (de Hoog *et al.* 2019; Revankar and Sutton, 2010).

1.5 Sampling

Water dynamics ensure that whatever pollutant reaches it will be diffused to enormous extents. Sampling water at one site is thus representative of a large volume. Sand, conversely, is patchy and thus sampling and representativeness are relevant subjects for setting up effective monitoring procedures (Brandão, 2019; Brandão & Harwood 2016; Vogel *et al.* 2017). Brewer, *et al.* (*2016*) demonstrated that single grab samples are representative of nothing else but the sample under analysis in the lab. That is the reason why an incremental approach (Incremental Sampling Methodology) for soil sampling, although rather complex, is highly recommended. In this method "*Incremental sampling begins with identification of an area (for shallow soil, nominally only a few inches deep) or significant volume (for deeper soils where thicker*

sequences are of interest) of soil to be sampled, which is referred to as a decision unit. A decision unit defines a population to be investigated and sets the scale at which one wishes to make an observation or determination. (…) Within the decision unit, increments of soil are most often collected in a regular grid pattern that spans the entire area of the decision unit. Nominally 30 increments are collected as a required minimum. (…) The increments collected should be of equal mass and should be collected in an unbiased manner. When combined together all increments will typically only total 1–2 kg. Replicates collected from the same decision unit allow statistical treatment of the analytical results." (Hadley *et al.* 2013).

For locations with no historical information, which may help decision making, Brandão (2019) recommends a combination of incremental sampling and previous knowledge of hotspots of microbial contamination to sample worst-case scenarios and thus render the sampling of sand relevant and representative of a beach. Should incremental sampling not eligible, an alternative has also been described as a composite of three equidistant grab samples combined, homogenised, and analysed as one sample only (Sabino *et al.* 2011). The latter option intends to represent the entire beach, unless spamming more than a few hundred meters in length, in which case, the beach should first be divided into areas and each area considered one beach, for sampling purposes.

1.6 Analytical approaches

The earliest publications on sand contaminants are in 1960 for dermatophytes by Schönfeld *et al.* and in 1975 for FIB, by Oshiro and Fujioka (1975). The first had as target of the study the direct exposure to dermatophytes present in beach sand (Health protection); the second, the bacteriological water quality of Hanauma Bay in Hawaii, currently Hanauma Bay Nature Preserve.

Due to the extreme human pressure by its use, the water quality of Hanauma Bay was difficult to maintain within acceptable levels. It has now been classified a nature reserve and closed on Mondays and Tuesdays, and open the remaining days, until 4 p.m., to allow the local ecosystem to recover without human pressure (City and County of Honolulu, 2021). Because of being a bay, full of marine wide-life and high use by tourism and local beachgoers, the water quality led the authors of the research publication to sample the shoreline water and sand, land runoff, mongoose, and pigeon droppings, to try to find the main causes of the surges of FIB. The samples were analysed for faecal coliforms, *Escherichia coli* and enterococci, and revealed that the major sources of the periodically high levels of these bacteria in the water were

contaminants of the beach sand, namely pigeon faeces. In this case, water quality was the main driver of the research. There are, thus, different motivations and possible parameters of study on beach sand microbiology, which implicate different analytical approaches and methods.

1.6.1 Two tiers

Solo-Gabriele *et al*. (2016) recommended a two-tier approach to sand quality monitoring, aiming to simplify the analytical approaches to a minimum of requirements. Some of the methods described in the literature were extremely laborious and costly which does not work well with routine analysis to respond beach management subsequent actions needing to be validated before further actioning. The authors recommend thus a fast routine analysis, able to be performed in water quality laboratories and to escalate to reference laboratories only when it is necessary to establish the source of an outbreak, or to investigate the source of a highly prevalent contaminating species.

1.6.1.1 Routine

Sabino *et al.* (2011) describe analytical methods for fungi, by extracting sand with water 1:1, in low energy, shaking orbitally, at 100 rpm, during 30 minutes, followed by plating triplicates. Malt yeast agar with chloramphenicol is used for all species and Mycosel agar (with chloramphenicol and cycloheximide), specifically for dermatophytes. The method follows with incubation of 5 and 15 days at 27.5 ºC, respectively, followed by tentatively identifying all colonies, picking, and counting one colony of each morphotype, and dividing them into yeast-like species, opportunistic and allergenic species, and dermatophytes as 3 parameters to assess fungi. The result is the average count of the triplicates for each parameter, per gram of sand. This method is rather laborious and time consuming but provides a full snapshot of the culturable mycobiota in a sand sample. For FIB, the same group does a 1:10 (sand to distilled water), for 30 min. circular shake, at 50 rotations per minute (RPM) extraction, followed by using IDEXX™ (IDEXX, Westbrook, MN, USA) Most Probable Number (MPN) Colilert® and Enterolert®, according to the instructions provided by the manufacturer. Boehm *et al.* (2009) recommends a faster approach by extracting 10g of sand with 100ml of distilled water or PBS, followed by membrane filtration method of the eluent as if it were water (International Standard Organisation, 2000). The Environmental Health Department of the National Institute of Health Doutor Ricardo Jorge (Portugal), a section of this team, has since 2015 redesigned the fungi routine analysis, to implement the recommendations of Solo-Gabriele *et al.* (2016). The parameters of the basic fungal analysis are now the total fungal count, *Candida albicans* count, other yeasts count, and detection of dermatophytes.

In case of an outbreak, namely the one reported by Brandão *et al.* 2020, the Reference Mycology Laboratory is engaged to identify the species of fungi relevant for the intended study. For this study, the fungal species isolated are typically associated with either vegetable matter (colonisers and pathogens) or with faecal contamination, which was indeed the cause of the outbreak. The authors also explain how the fungal community found might be of help in detecting the cause of a pollution event.

Other teams have considered analysing total microbiome, using Next Generation Sequencing (NGS) (Mohiuddin *et al.* 2017; Romão *et al.* 2017; Taylor and Kurtz 2020). This approach requires sophisticated analytical procedures and equipment, and the results are not comparable with culture-based methods. Despite only analysing the present DNA, thus detecting both viable and inviable individuals, it is a good tool for environmental forensic analyses, since it provides microbial community composition information that accumulated during a long period of time. However, the most successful species will be over-represented and may conceal the less represented taxa.

Boehm *et al*. (2009) carefully addressed the extraction method and influencing factors - the group ran a three-site comparative study, to establish the most effective method to extract and analyse FIB from sand. The group was composed by several laboratories in the USA and used samples from the following regions and beaches: California (Doheny Beach), Florida (Hobie Cat Beach) and the Great Lakes region (Michigan City). The samples travelled in preserving conditions across the laboratories composing the analytical group to assess reproducibility. The easiest extraction method determined with the maximum FIB recovery was a 2-minute handshake in "*phosphate-buffered saline or deionized water, followed by a 30s settling time, one-rinse step and a 10:1 eluant volume to sand weight ratio*. *The result was consistent across the sand compositions tested in this study but could vary for other sand types*." The study also revealed that some analyst-effect might arise from with this approach. Vigorous shaking of extractions cannot be used for fungal analysis since hyphae break into several new colony-forming units. Instead, an orbital shaking is advised in order to retain integrity of the hyphae extracted.

Information on routine analytical approaches for viruses (mainly molecular, but also some culture based), protozoa and helminths is extremely scarce so they shall not be addressed further in this section.

1.6.1.2 Outbreak and full population analysis

In the absence of historical data on culturable microbial flora from beach sand, a primordial full-population-exploratory analysis might be an attractive approach. It will shortcut years of collecting scattered fragments of data to eventually generate a beach profile and be able to decide what is ordinary and what is extra-ordinary and may require attention (Brandão, 2019). An FIB surge is a frequent example. In this case Microbial Source Tracking (MST) methods may indicate the biological community where the bacteria originate from, if humans, ruminants, seagulls, dogs, etc. MST can thus distinguish between run-off and direct deposition forms of faecal pollution. However, without a history of microbial on a site, a surge of whatever organism is impossible to confirm. Alternatively, parametric reference values may be used as guidance for never tested sands, but these are yet to be published by any regulating agency. WHO (*in press*) will be the first regulatory document with sand microbial parameters and reference values (for enterococci and Fungi).

In case of a beach associated outbreak detection, by any microbial agent, an epidemiological study should be conducted, as the one described in Brandão *et al.* (2020). In such an event, it is recommended to analyse all kinds of variables, one of them being if the beach sand might be the cause of the outbreak. In this study, bathing was excluded as an outbreak propagation fomite because some of the patients did not bathe. The only common denominator to the reported macular erythematous pruritic rash outbreak was sand. As the cause was unknown during the investigation, the study was conducted, employing Organic Chemistry approaches, as well as Inorganic Chemistry, Bacteriology (for FIB) and Mycology. The study shows how multi-disciplinary analytical teams might need to be engaged in such events.

Regardless of the cause of concern, mitigating actions should take place. In the case of human faecal pollution, there is always a concern of transmission of any residual-water-borne pathogen, like enteric viruses, pathogenic bacteria, high concentration of opportunistic fungi, parasites, and Anti-microbial Resistance Genes (ARG). Climate changes will exacerbate this concern by adding non-endemic pathogens that find new niches to thrive (Whitman *et al.* 2014; Fujioka *et al*. 2015; Weiskerger *et al.* 2014; Teixeira *et al.* 2020).

1.6.2 Fungal analyses

1.6.2.1 Isolation

Unlike yeasts that are mainly buddying cells, moulds occur as hyphae. If broken, any section of a hypha will start a new colony. It is thus truly relevant how to approach fungal analyses of sand, considering that there will always be a trade-off between aqueous extraction from sand

and breakage of the hyphae during the process due to vigorous agitation. As mentioned previously in 1.6.1.1 (Routine), Sabino *et al*. (2011) opted for orbital shaking with a speed of 100 rotations per minute. The same laboratory is still doing so today, followed by plating in Malt Yeast Agar, supplemented with chloramphenicol (0.05%/L), for all fungi and Mycosel Agar, supplemented with chloramphenicol and cycloheximide. The latter is used specifically for dermatophytes, given the growth speed reduction of fast-growing fungi, which allows dermatophytes to grow and be visible, instead of swamped by fast growing moulds.

In mycology, it is necessary to use a medium to recover as much of the fungi one wants to isolate from an environmental sample, as possible. Sabouraud with chloramphenicol is the traditional choice as it is not selective. Yet, samples with abundant presence of *Mucorales*, may require the use of a medium with Dichloran Glycerol Agar combined with Rose Bengal, to inhibit their exuberant growth (Henson, 1981). This is frequently the case with inland beaches due to the heavier presence of vegetable matter since many species of this Order are plant pathogens (Shtienberg, 1997). Coastal beach sands may also yield some isolates of *Mucorales* but usually only when highly contaminated with fungi from many species.

Incubation needs to extend long enough to allow as many fungal colonies as possible to become visible, but not long enough to have the faster growing ones cover the slower growing ones. Again, there is a trade-off, for isolating mixed cultures of unknown species from environmental samples: many dematiaceous fungi are slow growing, including dermatophytes and *Exophiala* spp. (Joseph, 2019), which cause phaeohyphomycosis. Conversely, *Mucorales* and *Trichoderma* spp. are extremely fast growers (Henson, 1981; Harman *et al*. 2004). Moreover, yeasts grow by cell division on a growth medium, not shooting out hyphal ramifications. Hence, moulds spread onto the culture media while yeasts' colonies have a more restricted growth. Also, there is competition between both groups. For example, the statins (Endo *et al.* 1979), used for pharmaceutical purposes in humans to lower cholesterol levels in blood, are a in fact a metabolite produced by moulds, intended to slow down the growth of the yeasts and even of other species of moulds. The metabolite interferes with the production of ergosterol, necessary for their growth (Macreadie *et al*. 2006). The inoculum from an environmental sample should thus be diluted enough to allow all species to grow without confluency, during enough time of incubation, and at a temperature that is appropriate for the intended purpose. However, dilution of the inoculum to ensure a robust analytical disposition of all colonies will inevitably lead to the loss of lesser present species (Franklin *et al*. 2001).

The sand analysis for health protection should target a temperature that matches that of the surface of the human body. The approach used in bacteriology of selecting the pathogens as the bacteria that can grow at 37ºC does not apply to Mycology. Keratinophilic fungi will by default infect keratinised tissue, thus skin, nails, and hair(s). a temperature of 27.5ºC will allow all medically relevant fungi to grow (Sabino *et al.* 2011).

1.6.2.2 Taxonomic classification

Fungal taxonomy is currently undergoing a revolution mainly due to new data arising from the use of molecular biology techniques (Money, 2013). Still, the primal approach tends to remain identification by micro and macro characteristics of the colony. Some of the common fungi found in the environment of the beach are readily recognisable at first glance, most of them needing a microscopic verification of the typical structures that may help to distinguish for example, some *Penicillium* and *Aspergillus* section *Fumigati* species. Yeasts, however, require always biochemical testing to be differentiated. Sabino *et al.* (2011) describe in detail, how a sand analysis can be performed also at the stage of identification of the fungi and bacteria; although, currently, the same institution identifies many of the fungi by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-ToF) or Molecular Biology based on sequencing of the ITS1 and ITS2 regions of the ribosomal Deoxyribonucleic Acid (DNA). MALDI-ToF is fast and requires little handling before a fine identification is achieved. Molecular identification tends to be broader in the number of species that can be identified but it is more costly and labour intensive (Brandão *et al.* 2021). This system allows the clear distinction of many cryptic species, depending on the primers chosen to perform the amplification of the target DNA.

1.6.3 Bacterial analyses

1.6.3.1 FIB

The FIB in sands are currently identified mainly with the use Colilert® and Enterolert® from IDEXX Laboratories, Inc. (Maine, USA). The principle is to extract the FIB by shaking the sand sample immersed in distilled water, followed by processing the extract as any water sample. The analysis returns the most probable number of CFU/ml of Coliforms, *E. coli* and enterococci, which must be reversed to the dilution used in the extraction.

1.6.3.2 Other bacteria

Bacteria, in general, may offer some resistance to isolation due to the level of contaminants that exist in an environmental sample. Nonetheless, selective media and temperatures are the usual methods. Following the same system as for FIB, Pseudolert® (also manufactured by

IDEXX Laboratories, Inc., Maine, USA) can be used for the detection and count of *Pseudomonas aeruginosa*. Other bacteria necessitate specific isolation techniques other than those manufactured by IDEXX. The scientific literature is scarce on bacteria other than FIB but in 2009, Goodwin and Pobuda, studied the presence of methicillin resistant *Staphylococcus aureus* (MRSA) by performing the following procedure: "*Sand was prepared for membrane filtration by vigorously hand shaking sand into PBS for 2 min using a ratio of 2 g sand to 80 ml of PBS (…). The solution of sand and dislodged particles was vacuum filtered through a sterile, 30 µm, 47 mm nylon net filter (Millipore, Bedford, MA, USA). An additional 10 ml rinse with PBS was used to remove any remaining sand from the shaking container. This procedure was repeated until a sufficient volume of "sandwater" was generated to satisfy the membrane filtration needs for that site. The "sandwater" was homogenized by hand mixing prior to filtration. Filters were incubated either on SCA or C-MRSA (BD Biosciences, San Jose, CA, USA). SCA is a selective and differential medium for S. aureus and C-MRSA is a selective and differential medium for MRSA."* (Goodwin and Pobuda, 2009).

1.6.4 Microbial Source Tracking (MST)

In case of a surge of FIB the inevitable question is… what was thy come from? Sand contaminated by water and vice versa has been extensively addressed in Whitman *et al.* (2014) - dispersion, survival, predation on, and, ultimately, the fate of the FIB in sand and water. But what is still under intricate discussion amongst the scientific community is how to find their origin. Naturally, FIB that indicate human excreta is a clear warning that human pathogens may be present and cause harm to humans. These are the most relevant FIB for human health but not the only ones. Other FIB or different biological groups may indicate possible zoonoses (like bird flu concerns), Haemorrhagic *E. coli*, and ARG. It is thus desirable to be able to track the origin of FIB in sand and in water during a surge, and hopefully mitigate it upstream.

In 2000, Bernhard and Field, developed a way to characterise faecal contamination from cows and from humans, based on the 16S subunit of the ribosomal DNA, of the genus *Bifidobacterium* and the *Bacteroides-Prevotella* group. They reported PCR primers that differentiate both these specific biological groups. The aim of their research was mainly to help identify and mitigate diffuse (non-point-source) water faecal pollution, which in wet regions of many parts of the most developed parts of the world tends to be associated mainly with run-off carrying faecal contamination from cattle. Currently there are many more sets of primers, covering several biological groups, including seagulls and dogs. The method is currently being tested to use also in contaminated sand, in the Department of Environmental Health of the

National Institute of Heath Doutor Ricardo Jorge, following the investigation of an episode of persistent faecal contamination of the sand of Praínha, Island of Terceira, Azores (Jornal Açores 9, 2019). The origin of the pollution was unclear and testing dogs was not implemented at that time, only for ruminants and humans. The local authorities suggested several possible contamination scenarios, one being dog walking at the beach after dark. Another, a contamination from below, from the phreatic (saturated) zone, which lies underneath the vadose zone. The other options were seagulls and waterfowl, or runoff from the city above or washup. Human primers did not amplify the DNA extracted from the contaminated sand samples, and neither did the ruminants. This excluded the contaminations by run-off from any direction possible. Eventually, the conclusion was that dog walking was the likely cause of the pollution, given the distribution of the samples with the highest levels of contamination along the beach extension coinciding essentially with beach access. Ultimately, the results of testing the uprise or deposition of the FIB, by isolating and covering a section with an area of four-square meter with an impermeable membrane, clarified the deposition nature of the contamination. After a few days of the sand patch being present after treating the sand with chlorine, the contamination in the beach sand was back but not in that particular section. The City Hall has since then installed vigilance cameras and put up very obvious signage of beach dog-walking not being allowed for public health reasons. This research is currently undergoing molecular investigation with dog *Bacteroides* primers, for forensic confirmation, before submitting for publication in a scientific journal.

Other methods for MST are also in use, namely using Next Generation Sequencing (NGS) based DNA analyses, but the costs are high and requires intense computer analyses. Two options available are the comparison of Operational Taxonomic Units (OTU) specific of biological groups of origin and shared to assess the level of contamination originating in humans and in other biological groups. The other is a "*Bayesian algorithm, to determine which OTUs have contributed to an environmental community based on the composition of microbial communities in multiple fecal sources*" (Unno *et al.* 2018). The authors of this publication state the following, concerning NGS methods for MST: *"Population-based MST methods are frequently library-dependent – they require de novo construction of a library of features associated with a particular source. Building a library thus involves the extensive characterisation of phenotypic or genotypic patterns (e.g. antibiotic resistance or rep-PCR genotyping) of a particular population (e.g. enterococci) associated with each source of interest (Stoeckel and Harwood, 2007). In order for successful classification of fecal sources,*

hundreds-to-thousands of isolates must be typed from each source of interest as well as from recreational waters with an often cumbersome investment in cost and labour. Furthermore, libraries are rarely applicable outside of a particular study area and are temporally variable (only relevant for a brief period), thus classification of sources is frequently imperfect (Harwood et al. 2014; Ishii and Sadowsky, 2009)." Not surprisingly thus, the method of choice that seems to be gaining territory internationally is Bernard and Field's method described above.

1.6.5 Quality Assessment Schemes

Once the sample of sand arrives at the laboratory, its analysis has several points that require finetuning to ensure reproducibility and repeatability which do not depend on the equipment used: the efficiency of the aqueous extraction of the sand, the plating of the extract, the plate reading and registering with the necessary calculations to produce a value of CFU/g. Mentioned under the "Routine" heading of section 1.6.1 (Two Tiers), Boehm *et al*. (2009), published an interesting article on sand bacterial analysis and performance of participating laboratories in an interlaboratory collaborative study. The authors found that there is a variation in blending, shaking, and by analyst. No analysts were rejected from the study. Instead, they contributed to establish the natural variation of the results. Rinsing, decanting, and settling before using the eluent did not show any statistically significant variation ($p<0.05$).

Participating in an interlaboratory assessment scheme is thus extremely relevant for sand analysis. It is the only tool that can ensure that the results obtained are independent of the analysts and also serves as a training tool. Many laboratory analysts work alone and only have an idea of how good they perform in terms of normality, by comparison with others working the same sample. This, on the other hand also has an informative rebound effect on assessing the validity of the scheme itself. Distributing samples for interlaboratory assessment schemes implies processing, transporting, and handling of samples until the end-user laboratory and the logistics involved might alter the sample in many ways. Non-refrigerated transport may either kill or permit multiplication of microorganisms. Processing and handling might contaminate samples. Analysis of the joint results will be informative of all these possible variations. The international standard used for determining a consensus value in proficiency testing by interlaboratory comparison is the ISO 13528 (International Standard Organisation, 2015).

1.7 **Water quality management**

As mentioned in section 1.6 (Analytical approaches), water quality is a strong driver for sand analytical monitorisation. A recreational area's local economies tend to be highly dependent of

the tourists. When beach management authorities need to close a beach for safety reasons, that local economy is brought down to a halt. For that reason alone, ensuring the good quality of a recreational water is crucial for all those relying on its uninterrupted use for a living. What happens when the results of the routine mandatory analysis on water quality fail to meet the regulatory requirements for recreational use? The local health authorities order their closure for public use until those results meet the requirements again. The WHO Guidelines for safe recreational water environments (2003) recommend a value of enterococci of 200UFC/100ml to ensure a no-higher-than-5% risk of acquiring a waterborne ailment associated with bathing. This value and parameter itself, however, are not necessarily the choice made by regulators who legislate the recreational water quality. For example, the European Union currently uses both enterococci (200UFC/100ml) and *E. coli* (500UFC/100ml) for inland waters and enterococci (100UFC/100ml) and *E. coli* (250UFC/100ml) for coastal and transitional waters, combined with a 95% percentile and a geometric mean to define a water of excellent quality, the kind that offers a low risk of contamination (5%) (EU, EEU, 2006). The epidemiological study that established these values was published in 1998, by Jay Fleisher and David Kay (Fleisher *et al.* 1998). It was later extended for inland waters, in Germany, by replicating the original study in inland German recreational waters (Wiedenmann *et al.* 2016).

As mentioned previously, the interaction between sand and water and how FIB travel within and in and out of water as well as their possible origin is extensively described in Whitman *et al.* (2014). More specifically, it explains how "*Allochthonous microbes, including fecal indicator bacteria (FIB) and waterborne pathogens, are deposited via waves, runoff, air, or animals. The fate of these microbes ranges from death to transient persistence and/or replication, to establishment of thriving populations (naturalization) and integration in the autochthonous community*". This article also describes the possible health hazards of associated pathogens, including fungi in sand. Weiskerger *et al*. (2020) describes the predictive modelling of dynamic water systems, which is considered the next upgrade in water quality management. Instead of looking at results of analytical results, collect them in time and space with enough frequency to be able to predict recurrent pollution events.

1.7.1 Point source pollution

The definition of point source pollution is when a source that comes from a single identifiable source. For example, when a contaminated creek ends at a beach. These are common situations and need to be worked on upstream. Sometimes, however, such water courses may travel partially under the surface, where in fact they may get their contamination due to contaminated

soil. This is often the case with chemical contaminated soils, even if the water course does not run under the surface at all. Nonetheless, the result is one only: a concentrated pollution distributary that will contaminate sand and water alike. The main difference between sand and water in this kind of pollution is that sand will act as a concentrator whereas the water will disperse the pollution. The use of predictive modelling in beach systems (as described in the previous section) will allow closing the beach preventively instead of too late for bathers, due to the time necessary to produce results in the lab and communicate them to the beach managers and local authorities.

1.7.2 Diffuse pollution

Diffuse pollution is the case when no specific tributary can be identified and therefore originates from soil infiltration or surface runoff. Sand and benthic sediments are usually involved in this process. Whitman and Nevers in 2003, demonstrated that the sandy shores acts as a bird pollution diffuser, receiving the bird droppings by deposition, concentrating it, and letting it be washed out into the water body, contaminating it diffusely. This kind of pollution is usually difficult to tackle since the source is very often difficult to control. When birds are the cause, one of the options described in the literature is to use biological control. Namely, dogs scaring away birds by chasing them through the beach and forcing them to settle elsewhere (Lubick, 2012). If the pollution source is phreatic there is not much hope to be able to correct at the source. Targeted methods may need to be implemented as described by the Organisation for Economic Co-operation and Development (OECD, 2017).

1.8 **Climate change and globalisation**

Climate change is a global changer. Climates dictate much of what is autochthonous in any location. The concept of endemic species is that these belong to a specific geographical setting and as such, are well adapted to the local climate and ecosystem. As climate changes, many of those species may migrate, no doubt often carried by traveling people or goods and settle elsewhere where the conditions are also favourable. Weiskerger *et al*. (2019) addressed this very issue extensively, focusing on the vadose zone. The rationale behind the publication is to contemplate changes that are expected to happen due to the current known changing scenarios. In this paper, desertification and increasing temperatures in certain areas of the globe will promote migrations of the human communities (and of many other species) to near water sources, leading to an increase in environmental pressure due especially to the more concentrated waste waters that need to be recuperated before releasing in the environment. The organisms of focus in the paper were *Vibrio* spp., *Staphylococcus aureus* and MRSA,

Leptospira spp., *Cryptosporidium* spp. and *Giardia* spp., *Candida* spp., Norovirus and Rotavirus and *E. coli*. The expectation of the authors is that all these may be of relevance, in terms of water and sand quality in the future.

In terms of pollution events, the biggest changers are the ever increasing in frequency and intensity extreme weather events: hurricanes, heatwaves, cold waves, extreme precipitation in any form, including large hail balls. The tropical storm hitting the island of Madeira in 2010, as described above in 1.3 (Sampling), was such an event and led to the destruction of many infrastructures build to contain and treat wastewaters. There was no record of an increase of GI illness following the storm, but it is also common for people suffering from a transient mild condition to not seek professional medical assistance Gibbons *et al.* (2014). The European Centre for Disease Control and Prevention (ECDC) logs all the known health effects of such cases in order to maintain a record for future studies and investigations (toolkit outbreaks). This was used during the preparation of Solo-Gabrielle *et al.* (2014) for which the records of any documented waterborne disease outbreaks were requested and thus provided for research purposes. The publication was meant to serve as a guide for sand microbiological approaches designed by a multi-disciplinary group and therefore a record of known waterborne diseases was desirable to help build the panel of microbes of interest. One of the most recent worries in Europe are *Vibrio* spp in several areas currently being monitored by the ECDC. Some are a common cause of GI illness in tropical areas, but others are flesh-eating and have been known to cause seriously destructive illness in inland water basins of central and northern Europe, as reported in the case described by Hirk *et al*. (2016).

1.9 AntiMicrobial Resistance/Genes (AMR/ARG)

Antimicrobial resistance is an emerging concern in water quality and much of what is found in water originates on land. Leonard *et al.* (2018) found that it was possible to isolate class A extended-spectrum β-lactamases gene carrying *E. coli* from 11 of 97 bathing sites of England and Wales. The extended use of antibiotics in several industries has led to the acquisition and selection of resistance genes by bacteria that thrive within human communities. More specifically there are 6 bacterial super pathogens of concern due to their multi-drug (MDR) and extensively drug (XDR) resistance. They are known in the water quality industry as the "ESKAPE" pathogens: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp. (van Goethem *et al.* 2018; Mulani *et al.* 2019). During swimming there is always a certain amount of water

ingested and therefore bathing waters are now becoming a possible means of transmission of ARG.

Another emerging concern are fungi. Research is being done to find azole resistant *Aspergillus* section *Fumigati* in the environment (Sabino *et al.* 2021; Snelders *et al.* 2008). The mutations more frequently associated with this setting of propagation of AMR occur in the gene *cyp51A* and in its promoter. It is thought that this specific AMR is originated in the environment, due to the intensive use of azoles in agriculture, and it has extended from the epicentre of its first isolation in the region of the Kingdome of the Netherlands and The United Kingdom to many other places in the world (Mellado *et al.* 2006). The expectation is that these will become ubiquitous and therefore the beach may well come be a possible fomite, due to run-off, via the water and/or sand; birds already are also propagators of this AMR (Melo *et al.* 2020).

Candida auris, the biggest emerging concern amongst medical mycologists, is a multi-resistant opportunist whose environmental niche is yet to be found. It is an expanding concern in hospital settings because it is rapidly becoming a problem in intensive care units everywhere. It thrives on severely debilitated patients and is almost impossible to control. Recently, a publication revealed that this yeast, which was originally described in South Korea in 1996 and in an ear infection in Japan, in 1997, is present, and very much alive, in salt marsh wetlands and sandy beaches of the Andaman Islands, in the Indian ocean (Arora *et al.* 2021). This shows that this species is tolerant to salt. The implications of this finding are not necessarily that the sea would be the natural environment of this species but that it survives in salt water. Interestingly, authors also found antimicrobial sensitive strains in this study. The fact that these islands lie in the gulf of Bengal, almost entirely surrounded by India, Sri Lanka, Bangladesh, Myanmar, Sumatra, Malaysia and Thailand and that India and Bangladesh are problematic countries in terms of residual water treatment and flow into the gulf does not exclude that the presence of this species may result from inland to sea contamination. It is also a microorganism of concern with climate change and increasing temperatures in wetlands since it is a thermo-tolerant organism.

1.10 Beach sand quality - current situation

Concerns about beach sand as a fomite for human infections and source of FIB in bathing waters has been published since the 1960/70's. The only time it became a regular activity was in Portugal in 2010, by the hand of the Portuguese Blue Flag programme, in collaboration with the National Institute of Health Doutor Ricardo Jorge and the Portuguese Environment Agency. Between 2006 and 2011, this cooperation led to the monitoring of beach sand all over the

country, and resulted in a robust publication, that provided information on stability and relevance of the monitoring, including the detection of some variables causing alterations in the normal results (Sabino *et al.* 2011): sampling before the bathing season yields higher counts of microorganisms; yeasts and dermatophytes indicate intensive human use; there is a microbial cumulative effect of the beach use throughout the bathing season; and lastly, there was a significant correlation between *E. coli* and *C. albicans* and between enterococci and *C. albicans*, which confirms the human nature of contaminants, since *C. albicans* is almost human specific. This research took place during the early days of the implementation of the current European bathing water directive. Since the end of the implementation in 2016 the results may have changed significantly due to the investment made by all the member-states in excelling the water quality of bathing waters by improving the treatment of residual waters. Since this programme came to an end, no other known monitoring programme took place in sand. Knowledge on this particular field, however, has improved immensely since then with publications arising from all over the world. It is thus high time that regulation is generated to help guide and set a common world-wide plan of action to integrate sand quality in beach management tools as a common legal framework with a common protocol to assess sand quality.

1.11 Main objectives and outline of the thesis

1.11.1 Aim and objectives of the thesis

Despite all the information gathered during the past 50 years, some relevant points were still missing and needed to be addressed, such as: fungi of medical relevance (to look for in sand and in water) - how many colony forming units are acceptable or normal per gram of sand; also, do we need to consider climatic changes in the composition of the sand microbiota? Does it alter with climate change? Is a consensual analytical procedure possible at this stage of knowledge? Under what premises? These questions directed my dissertation consisting of the following targets:

- Study the effects of a changing Earth on predicting microbial dynamics and human health risks in the beach water/sand continuum;
- Identify information on sand exposure that was missing in the scientific literature and fill in possible voids by reviewing literature and writing about it;
- Run a collaborative study on fungal diversity in the sand and water of recreational water bodies of Europe and Sydney, Australia;
- Propose a consensus in methods and parameters for sand safety management.

1.11.2 Structure of the thesis - publications

This thesis compiles seven papers (six published and one in press), each corresponding to a chapter (Chapters 2–8). These chapters are preceded by a general introduction to the topics focused on the papers (Chapter 1), followed by a general discussion, where the most important findings are integrated and debated (Chapter 9).

Chapter 1

Consists of a general introduction presenting a review on microbial life in beach sand and water that is relevant for public health protection at the beach. Several biological groups are contemplated, as well as the current knowledge on their persistence and survival in recreational water settings. Monitoring options and current lack of regulation or standard methods created an opportunity for this dissertation, whose technical chapters are expected to contribute towards a consensus regulatory path which should integrate adjustments to accommodate the changes created by climate change.

Chapter 2

Weiskerger, C. J., **Brandão, J**., Ahmed, W., Aslan, A., Avolio, L., Badgley, B. D., Boehm, A. B., Edge, T. A., Fleisher, J. M., Heaney, C. D., Jordao, L., Kinzelman, J. L., Klaus, J. S., Kleinheinz, G. T., Meriläinen, P., Nshimyimana, J. P., Phanikumar, M. S., Piggot, A. M., Pitkänen, T., Robinson, C., Sadowsky. M. J., Staley, C., Staley, Z. R., Symonds, E. M., Vogel, L. J., Yamahara, K. M., Whitman, R.L., Solo-Gabriele, H. M., Harwood, V. J. (2019). Impacts of a changing earth on microbial dynamics and human health risks in the continuum between beach water and sand. *Water Res.* 162, 456–470.
https://doi.org/10.1016/j.watres.2019.07.006

This chapter is dedicated to the expectations of alterations that climate change may implicate in public health protection at beaches (sand and water), in predicted scenarios of Global warming, climate change and extreme weather events. In this chapter, the team addressed several points: Human use of beaches, Pollution sources and sand-water interaction in the continuum or vadose zone, Microbial biofilms around grains of sand, Microbial transport mechanisms, Environmental stressers of microbial life in sand (radiation, temperature, starvation, predation and competition, dehydration), Modelling of the microbial interactions and transport between sand and water, emerging concerns related to climate change and lastly, Management for the future, considering all the variables addressed.

Chapter 3

Brandão, J. (2019). Microorganisms in Beach Sand: What Do We Still Not Know? In: Nriagu, J. (Ed.), Encyclopedia of Environmental Health. Elsevier, vol. 4, pp. 390–392. https://dx.doi.org/10.1016/B978-0-12-409548-9.11763-4

This chapter intended to address missing points on a previous chapter of the *Encyclopedia of Environmental Health*, authored by Prof. Efstratiou from the Aegean University. The difficulty in making sampling of beach sand representative of a beach was the first point discussed. There is not a lot of literature on this subject for beach sand but there is for chemical contaminations of soil. A tactical approach needed to be recommended to a method that can be performed routinely in many places, as sand quality analysis tends to require. Historical data, incremental sampling, routine parameters and worst-case scenarios were addressed. Another point was the difference between intrinsic, or resident microbiota and extrinsic microbiota, resulting from direct deposition, shedding, run-off and wash up. This point was followed by another with a brief discussion on risk analysis of exposure to sand at the beach, another on AMR expectations in beach flora, contributing to the insertion of AMG in the nearing communities, and a last one on global warming expectations and concerns.

Chapter 4

Brandão, J., Weiskerger, C. Novak Babič, M. (2020). Fungal Exposure and Relevant Recreational Settings. *Reference Module in Life Sciences*. (*in press)* http://doi.org/10.1016/B978-0-12-809633-8.21541-0

Fungi in recreational water settings is not new in swimming pools and spas. Dermatophytes have long been studied in such settings but not much more. This publication addressed other fungi, namely black mould, which are able to grow with very little nutrition and tend to resist chlorination, using thermal energy and electromagnetic radiation as energy source. They are thus strong survivors in any humid context. Beach sand is no exception, and the dark pigment of the cells, melanin, is the engine to harness solar radiation and use it to fuel the cell's energy needs. Are settings are also considered, like camping, children's sandboxes, Quantitative Microbial Risk Assessment (QMRA), a calculation of risk assessment from exposure, and climate change expectations of fungal contaminants in recreational water settings.

Chapter 5 – Restricted access – 5,676 words, 39,065 characters

Weiskerger, C. J., **Brandão, J** (2020). Fungal contaminants in water and sand: A new frontier for quantitative microbial risk assessment. *Curr. Opin. Environ. Sci. & Health*. 16, 73-81. https://doi.org/10.1016/j.coesh.2020.03.001

QMRA specialists usually address FIB and waterborne human pathogenic viruses but never fungi. Fungi are mainly opportunists and thus the risk of infection is very much dependent on the host, not so much the fungus itself; infectious doses are thus not easily determined and are a requirement of QMRA calculations. Nonetheless, there are two groups of fungi, the dimorphic/endemic fungi and the dermatophytes which are considered as real pathogens, not opportunistic. For these two groups QMRA should be done but rely on the estimation of a yet non-existing infectious dose. This publication aimed at raising awareness for fungi amongst QMRA specialists and epidemiologists who can help determine infectious dose. Some of the endemic fungi are highly compatible with recreational water settings in the Americas, like *Cryptococcus deuterogatii*, *Paracoccidiodes brasiliensis*, *Histoplasma* spp., and *Blastomyces dermatitidis*.

Chapter 6

Brandão, J., Gangneux, J.P., Arikan-Akdagli, S., Barac, A., Bostanaru, A.C., Brito, S., Bull, M., Çerikçioğlu, N., Chapman, B., Efstratiou, M. A., Ergin, Ç., Frenkel, M., Gitto, A., Gonçalves, C.I., Guégan, H., Gunde-Cimerman, N., Güran, M., Irinyi, L., Jonikaitė, E., Kataržytė, M., Klingspor, L., Mares, M., Meijer, W.G., Melchers, W.J.G., Meletiadis, J., Meyer, W., Nastasa, V., Novak Babič, M., Ogunc, D., Ozhak, B., Prigitano, A., Ranque, S., Rusu, R.O., Sabino, R., Sampaio, A., Silva, S., Stephens, J.H., Tehupeiory-Kooreman, M., Tortorano, A.M., Velegraki, A., Veríssimo, C., Wunderlich, G.C., Segal, E. (in press). Mycosands: Fungal diversity and abundance in beach sand and recreational waters - relevance to human health. (2021). *Sci. Total Enviro*. 781, 146598. https://doi.org/10.1016/j.scitotenv.2021.146598

Between 2018 and 2020 a pan-European team covering the Mediterranean, the Atlantic, the Black Sea, The Baltic Sea, The Adriatic Sea, The Italian Lakes and one in Sydney who covered three bathing sites of Sydney, including Bondi beach, collected samples, and processed them to explore fungi in water and sand of beaches of all these water bodies. The team sampled 91 bathing sites and processed 372 samples of sand (from 13 countries), and 315 of water (from 11 countries). The publication shows most of the data collected and processed by the following influencing factors, as decided by the team: Sand composition, Geographical location, Period

of the year, Urban and non-urban beach, Coastal and inland beach. For Fungi, the following parameters were studied: Yeasts (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 (all *Candida* species, including all species processed independently); 'Dermatophytes' (all identifications as *Microsporum, Trichophyton, Arthroderma, Epidermophyton* and (unidentified) Dermatophytes); 'Allergenic fungi' (all species of fungi excluding Yeasts and Dermatophytes); 'Dematiaceous fungi' (all fungi with melanin-like pigments in the cell walls); *Aspergillus* section *Fumigati, Aspergillus* section *Nigri, Aspergillus* section *Flavi, Candida parapsilosis* sensu lato, several *Candida* species, *Rhodotorula* spp, *Cryptococcus* spp and *Fusarium* spp. The study yielded many interesting values, one of which being a site-blind median number of CFU/g of 89 for any fungi in beach sand. This value has regulatory value as it serves as an indication for beach managers to consider as a normal value and has been included in the WHO guidelines (chapter 8), currently in press.

Chapter 7

Brandão, J., Albergaria, I., Albuquerque, J., José, S., Grossinho, J., Ferreira, F. C., Raposo, A., Rodrigues, R., Silva, C., Jordao, L., Sousa, M., Rebelo, M. H., Veríssimo, C., Sabino, R., Amaro, T., Cardoso, F., Patrão-Costa, M., Solo-Gabriele, H. (2020). Untreated sewage contamination of beach sand from a leaking underground sewage system - An episode of skin rash was experienced by 30 people at a beach. *Sci. Total Environ*. 740, 140237. https://doi.org/10.1016/j.scitotenv.2020.140237

In the end of June of 2019, 29 people using the beach of Porto Pim in the island of Faial reported a macular erythematous rash. Some never went in the water. An environmental and epidemiologic investigation was launched to identify the cause of the outbreak, in collaboration with the local authorities. Sand samples were taken from several locations along the beach, and it was established that the most contaminated samples were close to the access to the beach, where a bar is placed on top of a low cliff. The team that conducted the laboratory analysis performed bacterial and fungal analysis, organic chemistry, inorganic chemistry, and aromatic compounds. The combined results suggested a faecal contamination, which led the local authorities to find a degraded sewage distribution box that was leaking sewage into the beach sand, by gravity. The water was also slightly affected but not as extensively as the sand. The patients did not report any GI illness following the exposure. The irritating agent was found to

be sodium hypochlorite, used for cleaning the bar as preparation for the opening of the local bathing season a few days before.

Chapter 8

Brandão, J., Solo-Gabriele, M. H., Schets, F. M. (2021) Chapter 6 - Beach sand. *In*: WHO (2021). Guidelines on recreational water quality: Volume 1 coastal and fresh waters. Geneva, Switzerland (ISBN 978-92-4-003130-2).

In 2003, when the WHO guidelines for safe recreational water environments were issued, sand was one of the chapters – chapter 6. The authors and the leading organisation decided to refer to sand in the guidelines as one of the possible fomites encountered when visiting a beach. In 2020, a revised version of these guidelines was due, and a team was built to update chapter 6. Significant changes were made, compared to the 2003 version, and provisional parameters and levels have been put in, based for FIB in QMRA, and for Fungi on the value of CFU/g of sand found in Mycosands study (Chapter 6 of this thesis). The New guidelines will be made public during 2021.

Chapter 9

Finally, this chapter synthesises the main findings of the thesis, given the proposed objectives and a recommendation of methods for the provisional parameters established in chapter 8 is issued.

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Chapter 2

Impacts of a changing earth on microbial dynamics and human health risks in the continuum between beach water and sand

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Review

Impacts of a changing earth on microbial dynamics and human health risks in the continuum between beach water and sand

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ABSTRACT

Although infectious disease risk from recreational exposure to waterborne pathogens has been an active area of research for decades, beach sand is a relatively unexplored habitat for the persistence of pathogens and fecal indicator bacteria (FIB). Beach sand, biofilms, and water all present unique advantages and challenges to pathogen introduction, growth, and persistence. These dynamics are further complicated by continuous exchange between sand and water habitats. Models of FIB and pathogen fate and transport at beaches can help predict the risk of infectious disease from beach use, but knowledge gaps with respect to decay and growth rates of pathogens in beach habitats impede robust modeling. Climatic variability adds further complexity to predictive modeling because extreme weather events, warming

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water, and sea level change may increase human exposure to waterborne pathogens and alter relationships between FIB and pathogens. In addition, population growth and urbanization will exacerbate contamination events and increase the potential for human exposure. The cumulative effects of anthropogenic changes will alter microbial population dynamics in beach habitats and the assumptions and relationships used in quantitative microbial risk assessment (QMRA) and process-based models. Here, we review our current understanding of microbial populations and transport dynamics across the sand-water continuum at beaches, how these dynamics can be modeled, and how global change factors (e.g., climate and land use) should be integrated into more accurate beachscape-based models.

Contents

1. Getting our feet wet: introduction

Beaches are dynamic interfaces between shore, water and submerged bed, providing diverse microbial habitats that vary widely across space and time. Beaches play an important role in human recreational activities, supporting hundreds of thousands of jobs and generating billions of dollars in annual revenue (King, 1999). Each year, an estimated 140 million individuals engage in water-related recreational activities in the US (DeFlorio-Barker et al., 2016), providing opportunities for contact with beach microbial communities. Beachgoers expect the sand and water they encounter to be safe at beaches, which is not the case when sewage and other sources of fecal waste contaminate beaches with pathogens (Goodwin et al., 2012; Heaney et al., 2009; Korajkic et al., 2011; McQuaig et al., 2012; Sabino et al., 2011; Soge et al., 2009).

Humans exposed to feces-contaminated water and sand at beaches are at increased risk of infections that cause gastroenteritis, dermatitis, and other illnesses (Bonilla et al., 2007; Heaney et al., 2012). Human exposure to pathogens can occur as they interact with water and/or sand contaminated with pathogenic microorganisms from the waste of humans or other animal sources (Fig. 1). Epidemiological studies conducted in the US (USEPA, 2009) estimated the risk of acute gastroenteritis attributed to contact with water when swimming and wading to be 15 per 1000 individuals (DeFlorio-Barker et al., 2018; Wade et al., 2008; Wade et al., 2006; Wade et al., 2010). In the US alone, the annual cost of illnesses related to recreational water exposure is nearly $3 billion

Fig. 1. Schematic of the interactions between human beachgoers and the beach environment, potentially leading to exposure to pathogens. Image courtesy of Megan Smith, Jingru Chen, and Michael Winikoff at University of Minnesota.

(DeFlorio-Barker et al., 2018).

Worldwide regulation of recreational water quality is largely dependent on quantification of fecal indicator bacteria (FIB), such as fecal coliforms, *E. coli,* and enterococci (Parliament, 2006; USEPA, 2012; WHO, 2003), but sand is not routinely monitored. Many reviews have noted the pros and cons of using FIB as surrogates for pathogens in recreational water, including most notably their failure to correlate with pathogens (Boehm et al., 2009; Field and Samadpour, 2007; Harwood et al., 2014; Korajkic et al., 2018). Current understanding of the factors that influence relationships among FIB and pathogens in water is incomplete, and almost entirely lacking in sand. In water, FIB and pathogen levels are influenced by temperature, salinity, precipitation, sunlight location (water, sediment, vegetation), nutrients, microbial community structure, predation, desiccation, competition and contamination sources (Lipp et al., 2001; Medema et al., 1997; Wanjugi and Harwood, 2013; Whitman et al., 2008). The few studies conducted in sand habitats have found that moisture, microbial community structure, temperature (Eichmiller et al., 2014; Feng et al., 2010; Mika et al., 2009), stranded seaweed (wrack (Quilliam et al., 2014); and associated insects (Swinscoe et al., 2018) affect FIB and pathogen survival.

Epidemiological studies have shown that exposure to beach sand can increase the risk of gastroenteritis (Heaney et al, 2009, 2012) by activities that include hand-to-mouth transfer of microbiota (Whitman et al., 2009). Sand may also offer a transmission mode for opportunistic pathogens of non-fecal origin such as dermatophytes, which are shed by beach users (Anderson, 1979; Havlickova et al., 2008; Solo-Gabriele et al., 2016). Sand is a reservoir for hookworm species *Ancyclostoma spp* and *Necator americanus* (Yu and Blackburn, 2018) and the parasitic roundworm *Strongyloides stercoralis* (Boggild et al., 2016). Because of the expense of epidemiology studies and the impracticality of measuring all possible waterborne pathogens, quantitative microbial risk assessment (QMRA) has assumed an increasingly important role in estimating adverse human health outcomes from waterborne and other beach-associated pathogens (Ashbolt et al., 2010; Brandão et al., 2015; Haas et al., 1999; Jang and Liang, 2018; Schoen and Ashbolt, 2010; Shibata and Solo-Gabriele, 2012). Data generated through the QMRA process can also be used in the development of predictive models. The QMRA framework can help assess the impacts of changing environmental conditions at beaches and may enable more accurate management actions to prevent climate change-driven health impacts (Hofstra, 2011; Schijven et al., 2011; Sterk et al., 2013).

The challenges and costs of protecting public health from waterborne pathogens are continually increasing as population growth and urbanization increase pollution of surface waters from sewage and runoff (Liu et al., 2016). Climate change is altering temperature, precipitation, sea level, and storm intensities worldwide (IPCC, 2014; IPCC, 2018), with an estimated cost to utilities in the US of $448 billion to $944 billion (NAWA and AMWA, 2009). Given that these factors significantly influence the fate of waterborne pathogens and FIB, climate-associated impacts on microbial water quality are expected to be profound (Table 1; Burge et al.,

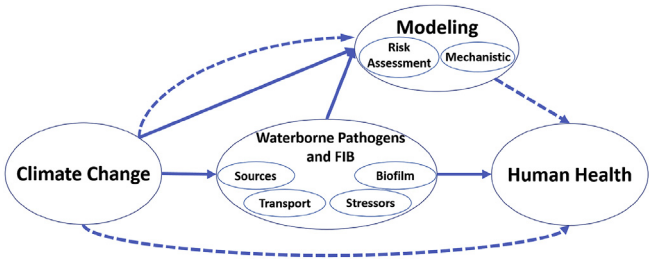


Fig. 2. Conceptual diagram of the relationsihps between climate change, microbial population dynamics, human health, and the models necessary to understand them. Solid-lined arrows indicate direct effects, while dashed-lined arrows show indirect effects.

Table 1
Examples of changing climatic conditions and predicted effects on selected microbes by geographic location and habitat.

Environmental Condition	Microbe	Predicted Effects	Study Site		Geographic Location	References
			Beach Sand	Recreational Water		
Increased Water/ Sand Temperature	*Vibrio* spp.	Increased environmental presence		●	Baltic Sea region, Germany, The Netherlands	(Baker-Austin et al., 2013; Huehn et al., 2014; Sterk et al., 2015)
	Staphylococcus aureus and MRSA	Increased persistence	●	●	California, USA	Goodwin et al. (2012)
	Leptospira spp.	Increased range and extended seasonality		●	Indian Ocean, China, The Philippines	(Desvars et al., 2011; Sumi et al., 2017; Zhao et al., 2016)
Altered Precipitation	*Cryptosporidium* spp. and *Giardia* spp.	Increased environmental presence with increased precipitation		●	New Zealand systematic review	(Britton et al., 2010; Young et al., 2015)
	Candida spp.	Increased presence with decreased precipitation (predominantly found in dry sands)	●		Portugal, Florida, USA	(Sabino et al., 2011; Shah et al., 2011)
	Norovirus and rotavirus	Increased environmental presence and persistence during extreme weather events		●	Brazil, USA	(McBride et al., 2013; Victoria et al., 2014)
Increased Wave Activity	*E. coli*	Elevated numbers in water correlated with increased wave height	●	●	Lake Huron, Canada	Vogel et al. (2016)
		Associated with wider dispersal of microbes and release into water	●	●	Lake Superior, USA	Ishii et al. (2007)
	Enterococci	Mobilized sand caused a spike in water contamination with increasing wave action	●	●	Florida, USA (experimental, laboratory conditions)	(Feng et al., 2013; Phillips et al., 2014)
		Positive association of microbe density in sand with wave height	●		Rhode Island and Alabama, USA	Heaney et al. (2014)
		Increased wave activity associated with wider dispersal of microbes	●		North Carolina, USA	Gast et al. (2011)

2014; Coffey et al., 2018; Dvorak et al., 2018; Patz et al., 2008). The imperfect relationship between FIB and pathogens hampers our ability to estimate human health risk from FIB levels; climate change and associated alteration of microbial habitats may make these relationships even more difficult to characterize. Thus, the existing pressures from human population growth coupled with predictions of further climate change have strong potential to amplify challenges in managing microbial pathogens in the beach sand-water continuum in the 21st century (Islam et al., 2018).

To protect human health at beaches, it will be crucial to address these challenges by characterizing and predicting their impacts using modeling frameworks like quantitative microbial risk assessments and hydrodynamic coastal models (Fig. 2 Coffey et al., 2014). Climate change has the potential to influence levels of waterborne pathogens and FIB by increasing pollution inputs, and by altering transport pathways and environmental stressors. Increased concentrations of waterborne pathogens will in turn have direct, negative effects on human health. Climate change will affect mechanistic models used to predict health risk at beaches directly, with altered ranges for physical-chemical data, and indirectly, by its direct effects on FIB and pathogen levels. Changing relationships between pathogens and the FIB used to predict them will directly affect predictive models of human health effects as a result of recreational water use.

This review synthesizes the anticipated impact of climate change and other anthropogenic effects on the ecology and human health implications of FIB and pathogens in the sand-water continuum. We focus on transport pathways from sources to beaches, habitats within the sand-water continuum, movement between sand and water, and persistence within the sand-water continuum. We present conceptual and mathematical models of microbial behavior in sand and use the models to discuss the potential implications of global change for human-pathogen interactions at beaches.

2. Pollution pathways: sources of fecal microorganisms

Pathogens and FIB enter beach habitats via both (1) direct fecal deposition from humans and animals at the beach, and (2) flow of surface water, stormwater runoff, or groundwater that has been contaminated elsewhere (Fig. 3; Cahoon et al., 2016; Hellberg and Chu, 2016; Hofstra, 2011; Kelly et al., 2018). Once present, extra-intestinal habitats such as aquatic macrophytes and sediments can also facilitate persistence and growth, which can then reintroduce microorganisms to the water column (Badgley et al., 2011; Beckinghausen et al., 2014; Whitman et al., 2003). Global change factors including altered human, animal and macrophyte populations (Chen et al., 2011), altered hydrology (Verhougstraete et al., 2015), and changing wastewater infrastructure (Abreu et al., 2016; Kessler, 2011; O'Mullan et al., 2017) can influence loading of FIB and pathogens to the sand-water continuum (Barreras et al., 2019; Liao et al., 2015).

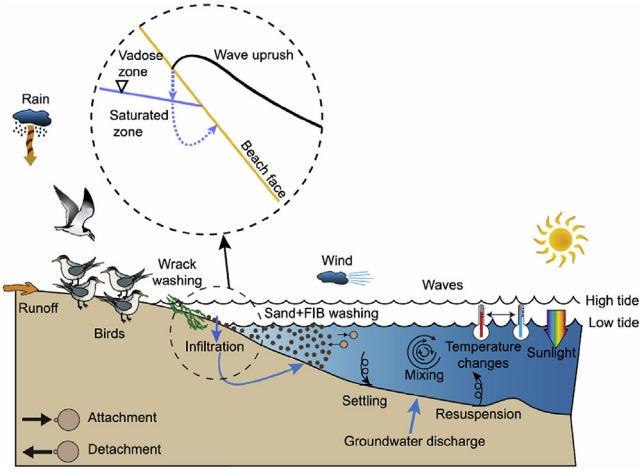


Fig. 3. Conceptual diagram showing key processes that influence FIB levels in the nearshore area. Not shown are environmental factors such as salinity, pH, dissolved oxygen (DO), or dissolved organic carbon (DOC) in the water column, which could modulate many of the processes illustrated.

2.1. Direct inputs & deposition to beaches

Human fecal contamination can be directly deposited to the beach environment via leaking beach sanitation infrastructure, stormwater and wastewater treatment plant outfalls, especially in the case of combined sewer overflows in extreme rain events or wastewater treatment plant failures, accumulation of solid waste such as diapers, or by bather shedding (Cahoon et al., 2016; Edge et al., 2018; Kessler, 2011). In addition to problems with aging sanitation infrastructure and lack of sanitation infrastructure, climate change is also expected to increase the frequency of wastewater treatment plant failures and combined sewer overflow (CSO) discharge to surface water during extreme rain events (Charlton et al., 2018; Kessler, 2011; Trtanj et al., 2016).

Changes in climate, human demographics, or cultural practices leading to increased bather density may increase risk for beach goers (Coffey et al., 2018; Schoen and Ashbolt, 2010), as a number of studies have linked bather shedding of fecal material and skin-associated microorganisms to pathogen levels (Elmir et al., 2007; Graczyk et al., 2010; Plano et al., 2011). Higher air temperatures and more extreme heat events may increase crowds at beaches seeking relief (Moreno et al., 2009; Smith, 1993). Expansion of recreational use at beaches may be particularly prominent near urban centers, due to population density and urban heat islands, leading to increased pathogen loading in beach environments from direct shedding, compared to less urbanized areas (Perkins et al., 2014; Shuval, 2003).

Direct fecal contamination of beach water by animals can result in increased levels of FIB and zoonotic pathogens, such as *E. coli* O157:H7 (Garcia et al., 2010). Human health risks from animal fecal contamination can vary, but many animal host species frequently carry human pathogens, such as cattle, poultry, and other birds (Brown et al., 2017; Garcia et al., 2010; Kinzelman et al., 2008; Soller et al., 2010). Some animals, including birds and rodents, can be sources of bacteria and fungi to sands and may cause skin infections like ringworm (Brandão et al., 2002). Climate change can affect regional bird abundance, as observed in northern Europe, which may lead to bird-associated problems at previously unaffected beaches (Virkkala and Lehikoinen, 2017). For example, gull and Canada goose populations are growing in many urban settings around the world and particularly around the Laurentian Great Lakes (Marzluff, 2001; Shochat et al., 2010). Livestock agriculture, which is a major source of microbial pollutants to water, is rapidly intensifying as human populations grow and demand the benefits of the industrialized world (Mateo-Sagasta et al., 2017).

2.2. Indirect inputs to beaches from water

Fecal contamination of beaches often occurs indirectly via inputs of water contaminated upstream. At almost any beach this can include surface water flow from nearby streams, rivers, or estuaries (Molina et al., 2014; Nevers and Whitman, 2005; Staley and Edge, 2016). Many beaches receive stormwater runoff from urban settings, whether directly via sewer and stormwater outfalls or

indirectly through overland flow. This runoff can be contaminated with FIB and pathogens by CSO events, leaking sewage or cross connected stormwater infrastructure, and impervious surface runoff (Marsalek and Rochfort, 2004; Nevers and Whitman, 2005). Defective septic systems and some wastewater infrastructure leaks can also contaminate groundwater that can then flow to the beach environment (Bishop et al., 1998; Foster and Chilton, 2004; Kracht et al., 2007; Rutsch et al., 2008). In fact, microorganisms can be transported in the subsurface for considerable distances under certain conditions (Ahmed et al., 2005; Arnaud et al., 2015); the effects of this contamination source are likely to increase with increasing urbanization and aging infrastructure. Agricultural runoff can also contribute high loads of ruminant fecal contamination and zoonotic pathogens to adjacent water bodies via runoff from concentrated animal feeding operations (CAFOs), grazing fields, or following manure application (Harmel et al., 2010; Islam et al., 2004; Jamieson et al., 2002; Jones et al., 2013; Niu and Phanikumar, 2015; Quilliam et al., 2011; Staley et al., 2013).

Predictions of changing precipitation amounts and intensities (IPCC, 2014; IPCC, 2018) are expected to directly alter the transport of pathogens and FIB from upstream sources to coastal waters and beaches (Ackerman and Weisberg, 2003; Curriero et al., 2001; Fowler and Hennessy, 1995; Hellberg and Chu, 2016; Hofstra, 2011; Mearns et al., 1995; Mimura, 2013; Patz et al., 2008; Trenberth, 1999). The increased storm intensities predicted for many regions may also result in greater resuspension of FIB from beach sand and sediment into the water column. Subsequent effects on water quality can last up to 5–7 days after the event (Ackerman and Weisberg, 2003; Curriero et al., 2001; Williamson et al., 2017). At the other extreme, increased drought may also alter human exposure to microbial pathogens by decreasing the dilution of pollutants, and by forcing consumption of contaminated water (Boyle et al., 2013; O'Dwyer et al., 2016; RIVM, 2010). Drought conditions may also favor persistence of certain pathogens and opportunists, including the fungal group *Candida*, which can survive in dry sands (Sabino et al., 2011; Shah et al., 2011).

The multiple direct and indirect pathways leading to the sand-water continuum complicate efforts to predict the magnitude of change in human health risk posed by anthropogenic change. Extreme storm events, intensification of agriculture, and increased urbanization will all contribute to increased levels of FIB and pathogens transported to beaches. Deterioration of the sanitary quality of beaches, and greater subsequent exposure of humans to waterborne pathogens are very likely without changes in current human and animal waste management practices.

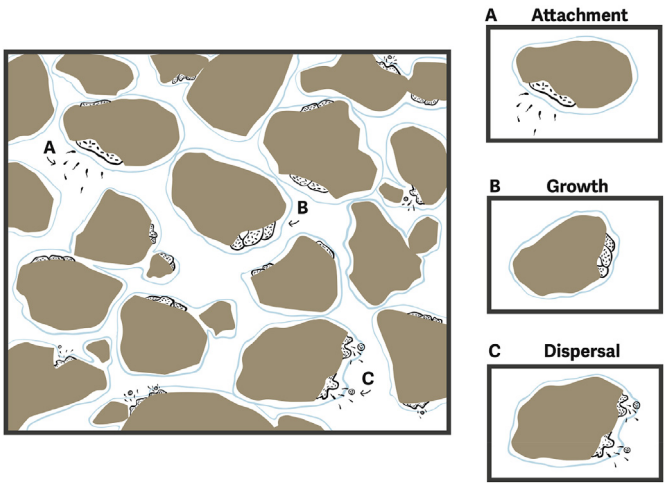


Fig. 4. Biofilm stages and dynamics within the context of sand at the sand-water interface. Image courtesy of Jingru Chen at University of Minnesota.

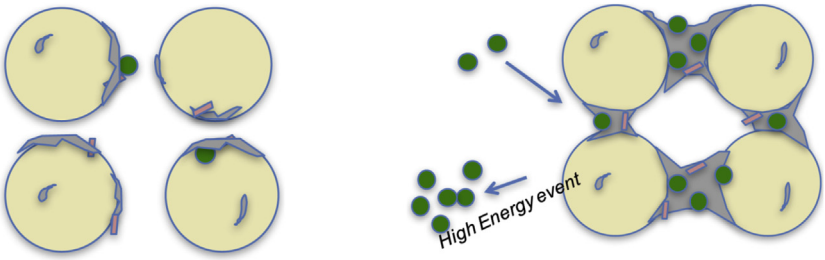
3. Hangouts: biofilm habitats in sand

Most microbial habitats, including sand, are dominated by biofilms: multispecies communities attached to dry or wet surfaces, or even at the water-air interface (Fig. 4; Almatroudi et al., 2015; Tan et al., 2017). *E. coli* and enterococci form sand-associated biofilms (Phillips et al., 2011b; Wang et al., 2011), and there is every reason to expect that waterborne pathogens also survive there. Pathogenic bacteria such as *Pseudomonas aeruginosa* survive otherwise inhospitable environments such as drinking water in biofilms (Mena and Gerba, 2009). Known biofilm inhabitants like *E. coli* can firmly attach to sand grains via biofilms (Wang et al., 2011; Whitman et al., 2014). One study consistently found pathogen genes *eaeA* (enteropathogenic *E. coli*) and *pic* (*Clostridium perfringens*) in beach sand (Zhang et al., 2016).

Biofilms benefit inhabitants by providing access to nearby nutrients and protection from chemical and biological harmful agents such as antibiotics (Balcazar et al., 2015; Costerton et al., 1987) and protozoa (Matz et al., 2004; Weitere et al., 2005). Surface charges and extracellular polymeric substances (EPS) secreted by bacteria mediate surface attachment and help create microenvironments in which oxygen levels and nutrient gradients contribute to complexity of the community. The close proximity between biofilm members contributes to quorum sensing (microbial talk) and altered gene expression (Jayathilake et al., 2017) as well as the potential for horizontal gene transfer.

At fluvial beaches, flow is dominated by seasonal fluctuations rather than tidal impacts, and biofilms are rather unexplored including the complex communities formed by algae, cyanobacteria, fungi, bacteria, and protozoa embedded in a dense EPS matrix were identified (Corcoll et al., 2012). Images of bacterial biofilms assembled on sediments of a beach in Alentejo, Portugal show dense EPS and a close association between phytoplankton and bacteria (Fig. S1; Jordao, 2016). Besides FIB, *Enterobacter cloacae* (Mezzatesta et al., 2012), *Klebsiella pneumoniae* (Percival et al., 2015), *Acinetobacter baumanni* (Antunes et al., 2014) and *Aeromonas spp.* (Skwor et al., 2014) have all been described in this kind of matrix. But the full extent to which human pathogens persist in biofilms in beach sand is largely unexplored. This constitutes a significant knowledge gap that hinders the effort to estimate human health risk from contact with sand (Whitman et al., 2014).

Biofilm structure and EPS composition are related to both the bacterial species and the surrounding environment. Biofilms can behave as both viscoelastic solids and liquids (Fig. 4; Fabbri et al., 2017; Peterson et al., 2015), allowing them to structurally deform under various shear stresses and recover when the stress is removed. If forces such as wave or tidal action overcome the viscoelastic biofilm, portions will detach and ultimately be released into the water column (Fig. 4; Boehm and Weisberg, 2005; Phillips et al., 2014). One conceptual model for biofilms associated with beach sand proposes that biofilms build up and concentrate FIB and other fecal microorganisms under low energy conditions. The onset of higher-energy conditions, such as a tidal surge or high wave activity, may release the microorganisms from the sand biofilms and into the adjacent surface waters. Microbial settling and accumulation would be prevented at high energy beaches by constant removal or dilution through hydrodynamic mixing and sediment transport (Fig. 5). Feng et al. (2016) demonstrated that the frequency of exceedance of water quality standards based on FIB levels was negatively correlated with long-term mean wave energy at Florida, USA beaches. A body of evidence indicates that beaches with low waves have higher FIB levels than beaches with high



High energy time (winter): Sand has less time in the "resting" state due to higher wave energy. Smaller or compositionally/structurally different biofilm communities can develop, making FIB survival/attachment more difficult.

Low energy time (summer): EPS/biofilm can develop. FIB integrate into the biofilm community until an increase in energy sloughs the biofilm and FIB off the sand and resuspends them into the water column

Fig. 5. Conceptual model of biofilm development and FIB attachment/release as it relates to wave energy. Images depict model sand grains with biofilm and FIB (green circles) incorporated into the community. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

energy waves (Abreu et al., 2016; Donahue et al., 2017; Piggot et al., 2012; Yamahara et al., 2007). Wave energy is influenced by beach bottom slope and seasonality (Fig. S2). The seasonal nature of wave energy, which is higher in winter, can promote a greater accumulation of EPS (a surrogate for biofilm quantity) during September (summer) versus February (winter) (Figs. 5 and S2).

Given the dynamics between biofilms and wave energy, a conceptual model that describes the FIB release from biofilm in sand, $X_{fib,\ sand}$, (in units of numbers of microbes per time) could resemble the following:

$$X_{fib,\ sand} = \mathrm{f}\,(T, M, r_{eps}) \quad (1)$$

Where T is the time since the last energetic event was greater than 1 standard deviation of the mean wave height, M is the magnitude of the event above the mean wave height, and r_{eps} is the rate of EPS deposition on sand (in units of mass per time). T will generally vary, e.g. T at a given beach may be smaller during the storm season and larger in dry periods, resulting in seasonal variation in biofilm-associated FIB. The magnitude of the wave heights, M, may also influence $X_{fib,\ sand}$, with greater M leading to more reworking of the sand, more turbulence and more sand shear, minimizing the degree to which biofilms can accumulate. Under controlled experimental conditions within a wave flume, only 60% of enterococci were removed by waves with M values of up to 10 cm, suggesting that very large waves would be necessary to completely eliminate the enterococci reservoir from sand (Phillips et al., 2014). Vogel et al. (2016) suggests that *E. coli* are also released to surface waters during high intensity wave events.

The accumulation of EPS, r_{eps}, is influenced by the type of pollution (point vs. nonpoint source), as well as sand grain size and mineralogy. Vogel et al. (2017), Haack et al. (2003), and Skalbeck et al. (2010) observed that coarse sands have lower FIB densities, compared to fine sands. There also appear to be relationships between sand mineralogy and the attachment of EPS, and subsequently, FIB to sand grains, though there is debate over the nature of the relationships. Only 3% of the total sand FIB were released through pore water volume flushing in south Florida sand (Phillips et al., 2011b) as compared to nearly 100% at a California study site (Yamahara et al., 2007). Similarly, Hernandez et al. (2014) observed that calcium carbonate particles retain EPS and FIB more readily in comparison to quartz at a renovated beach. However, other cases have reported that mineralogy may not influence FIB releases from sand, such as is the case reported by Abreu et al. (2016) for beaches in Madeira, Portugal.

Given the importance of wave action in microbial resuspension from sand, increasing surface water temperatures (O'Reilly et al., 2015; Sharma et al., 2015; Wu et al., 2012), wind speeds, wave heights, and storm frequencies in coastal regions may decrease the diversity of microbial communities and thus make the coastal ecosystems more prone to disturbances (Byrnes et al., 2011; Tokinaga and Xie, 2011; Young et al., 2011). Storm intensity and frequency increases may lead to shoreline erosion and the development of flat, low wave energy areas (Wu et al., 2017), as detailed in 4.2 Waves and Tides. These changes, coupled with biofilm dynamics, could alter retention and exchange of FIB between sand and water along the coast, leading to changes in beachgoer risk. Increases to air and water temperature can also alter microbial communities in both the water and sand, expanding ranges of pathogens such as *Vibrio* spp., *Leptospira* spp. and *Salmonella* spp. poleward and into more temperate shoreline systems (Baker-Austin et al., 2013; Sumi et al., 2017; Viau et al., 2011). New experimental data are needed to assess the controlling factors in the retention, growth, and release of microbes from beach sands and sediments, and subsequent impacts to human beach visitors.

Biofilms play a vital role in the "hangouts" of FIB and pathogens in sand by shielding whole multi-species communities from external factors and protecting propagules that will withstand erosion associated with high energy waves. The effects of climate change on sand biofilms remain largely unexplored but may influence FIB and pathogen levels via higher temperature, which may alter microbial community structure, and physical factors such increased wave energy that may come with changes to frequency and intensity of storm events.

4. Cruising: mechanisms of transport

Within the beach system, various mechanisms mediate microbial movement between surface water, sand, and groundwater. Beyond local resuspension, FIB and pathogens may also be transported through the beach sand matrix and across the sand-water continuum via two general pathways: through-beach or over-beach. Through-beach transport of FIB, including their distribution in the subsurface and potential transport to surface waters via groundwater discharge is governed by infiltration and exfiltration (water flowing into, through and out of the unsaturated and saturated portions of the beach face), interstitial flow, and interactions with sediments, including attachment, detachment and straining (Bradford et al., 2014; Brown and Boehm, 2016; Molnar et al., 2015; Solo-Gabriele et al., 2016). The over-beach transport pathway is mainly associated with erosion of beach sand, which occurs in response to waves, tides, rainfall, and human actions such as beach

grooming, and FIB detachment that delivers microorganisms to adjacent surface waters (Vogel et al., 2016).

4.1. Physicochemistry

The texture and mineral composition of sand and sediment particles affect both groundwater flow and microbial transport because of relationships with pore size, hydraulic conductivity, and aggregate structure. Overall, beach sands are relatively unstructured and less conducive to macropore formation (Abreu et al., 2016), but transport can still be short circuited by preferential flow when varying particle size results in paths of higher hydraulic conductivity through the same matrix (Wang et al., 2013). Overall, however, in the absence of significant preferential flow, pore-scale mechanisms driven by interaction energies between the charged surfaces of organisms and soil particles become increasingly important, and can dominate in beach systems.

4.2. Waves and Tides

Waves drive the transport of FIB within the beach environment, by changing bottom shear stress (Chao et al., 2008; Thupaki et al., 2013) and playing a role in the resuspension of sand- and sediment-bound FIB (Gao et al., 2015; Thupaki et al., 2013). Waves lead to infiltration of large quantities of surface water and associated constituents (e.g., FIB and nutrients) across the beach face, particularly in the swash zone (area of wave run-up; Malott et al., 2017; Robinson et al., 2018; Xin et al., 2010). They drive rapid infiltration-exfiltration across the unsaturated and saturated portions of the beach face in the swash zone at a frequency of seconds (Heiss et al., 2015), as well as driving deeper interstitial flow circulations through saturated sand (Longuet-Higgins, 1983; Malott et al., 2016).

Tidal fluctuations can also drive large amounts of water flux across the beach face, with potential to transport FIB across the sand-water continuum. Tide-induced surface water infiltration generally dominates in the upper intertidal region and exfiltration dominates towards the low tide mark (Robinson et al., 2007). Gast et al. (2015) observed that microspheres, which were used as surrogates for bacteria, were transported from their initial location (0.05 m below the sand surface, just below the predicted high tide line) vertically to the groundwater table by tide-induced infiltration. Some studies, however, have shown higher concentrations of FIB in supratidal sands, above the high tide mark, compared to sands in the intertidal zone where greater infiltration and vertical transport of FIB into the beach matrix is expected to occur (Abdelzaher et al., 2010; Enns et al., 2012; Phillips et al., 2011a; Whiley et al., 2018). In addition, using shotgun sequencing, Mohiuddin et al. (2017) showed a greater taxonomic diversity in the supratidal sand than in adjacent surface water and Staley and Sadowsky (2016) showed that backshore sands had microbial communities distinct from those in nearshore sands. These findings may be because of lower moisture content in the supratidal sand compared to intertidal sands, which limit the survival of protozoan predators (Whitman et al., 2014), along with various endogenous sources such as bird fecal droppings (Brown and Boehm, 2016).

4.3. Precipitation

Rainfall events can also facilitate movement of FIB within the beach environment. Direct runoff across the beach surface may lead to over-beach transport of FIB toward the shoreline, and in some cases to the surface water (Silva et al., 2014). For example, Beversdorf et al. (2007) and Heaney et al. (2014) both showed that FIB were washed from the sand to the surface water by rainfall, with increases in observed surface water FIB and decreases in FIB levels in the sand. Alternatively, infiltration of rain through unsaturated sand surfaces may deliver FIB to the subsurface, and to the water table, where they may be transported via through-beach mechanisms (Russell et al., 2012). Silva et al. (2014) suggested that over-beach rather than through-beach transport was the most plausible mechanism during rainfall events at a freshwater beach.

In summary, microbial transport in the beach environment may proceed via over- or through-beach routes, and is influenced by matrix composition and type, precipitation, wave and tidal action. Interactions between microorganisms and particles are particularly important in through-beach transport. Waves and tides drive infiltration and exfiltration of water into the beach subsurface, and erosion of sand mediates detachment of microorganisms from particles, allowing transport of microorganisms to surface waters. Precipitation in the form of rainfall or snowmelt can mediate over-beach transport of microorganisms to surface waters; as such, precipitation-associated impacts of climate change are likely to alter microbial transport dynamics at beaches.

5. Bummers: environmental stressors

The diverse microbial community in beach sand must survive a wide range of biotic and abiotic factors affecting its growth and survival. Significant fluctuations of nutrients, competitors, moisture, and temperature influence the survival and composition of the microbial community, creating a complex spatial and temporal structure (Winfield and Groisman, 2003). Such fluctuations and their effects on the FIB-pathogen-opportunist communities in the sand-water continuum are integral to effective modeling/prediction of FIB fate and transport as well as nearshore water quality. In general, microbial levels measured by culturing are influenced more rapidly and profoundly than those obtained by molecular methods (Korajkic et al, 2018, 2019). The public health implications of method-related discrepancies in decay rates of FIB and pathogens in the environment have not been determined (Korajkic et al., 2019). Other substantial knowledge gaps exist, particularly in the context of differences between sand and water environments and impacts of climate change on interactions between biotic and abiotic stressors.

5.1. Nutrient availability and starvation

Nutrient availability in sand can be extremely limited, creating potential starvation conditions for gut microorganisms in extra-intestinal and environmental settings. Conversely, in some cases delivery and accumulation of particulate and dissolved nutrients to pore water via infiltration and exfiltration across the beach-water interface can lead to enriched microbial populations, including the potential increase of pathogenic organisms such as *Campylobacter*, *Salmonella*, and *E. coli* O157:H7 (Williams et al., 2007). Other nutrient sources, such as beach wrack (stranded macroalgae; Imamura et al., 2011; Quilliam et al., 2014), attached algae and aquatic vegetation and phytoplankton (Byappanahalli et al., 2006; Chun et al., 2015) can also enhance microbial survival and growth (Lim and Flint, 1989). The nutrient content in sand and sediment can also vary between climatic regions, leading to differential survival of FIB in tropical versus subtropical and temperate environments (Coffey et al., 2018).

5.2. Biotic interactions

FIB, pathogens, and opportunistic microorganisms in beach sand also face the threat of predation by microfauna, including protozoans and nematodes. While these microscopic organisms

have the potential to impact bacterial growth (Alm et al., 2006; Hartz et al., 2008), the composition of potential predators is not well-understood. An important factor in water (Wanjugi and Harwood, 2013), predation effects may be less notable in sand. Some studies identified protozoans as contributing significantly to bacterial decline in sand filters (Bomo et al., 2004), but predation has been shown to play a minor role in regulating the population size of *E. coli* and *Enterococcus faecalis* in natural sands (Feng et al., 2010; Mika et al., 2009). Similarly, the impacts of protozoa on FIB decay rates were negligible in tropical sediments from Singapore, and protozoa presence was not correlated with FIB presence or concentration (Nshimyimana, 2017).

5.3. Desiccation and solar radiation

Moisture levels across beaches vary in response to changes in precipitation, wave activity, tides, lake levels, hydrology, and meteorological conditions, as such the wetted beach zone can shift hourly. While fecal microbiota are abundant in the wetted foreshore (Whitman and Nevers, 2003; Wright et al., 2011), they are less abundant but still recoverable above the high tide mark (Abdelzaher et al., 2010) and in dry sand of freshwater beaches (Byappanahalli et al., 2006; Staley et al., 2015). Decreased moisture and increased sunlight have deleterious effects on FIB in sands (Caro et al., 1999; Mika et al., 2009) and, more broadly, moisture availability impacts microbial populations and communities by influencing microbial predation, nutrient availability, and viability (Mika et al., 2009). UV radiation is also major factor in *E. coli* inactivation (Jang et al., 2017; Whitman et al., 2004). Organic matter can mitigate this stress, as humic substances can shield microbes against UV radiation and facilitate moisture retention (Monteith et al., 2007; Weyhenmeyer et al., 2016; Williamson et al., 2017).

Excessive moisture in sand habitats can, however, be detrimental to microbial survival, and many bacteria, pathogens, and opportunists in the sand-water continuum require a water activity value within narrow ranges. Shah et al. (2011) found an inverse relationship between moisture content and survival of FIB, yeasts, and nematodes. Eichmiller et al. (2014) found that elevated moisture significantly impacted decay rates of microbial markers and pathogens, with slower decay occurring at 14 versus 28% moisture.

5.4. Temperature

Effects of temperature on microbial survival and growth, as well as members of autochthonous microbial communities are complex, differing by species and habitat, and whether microorganisms are measured by culture or molecular methods (reviewed in Korajkic et al., 2019). For example, optimal growth of *E. coli* and enterococci was observed between 23 and 32 °C and temperatures >50 °C reduced their levels (Beversdorf et al., 2007; Ishii et al., 2007; Mika et al., 2009). Cold temperatures can prolong microbial persistence and some fecal microorganisms survive freezing conditions (Francy et al., 2003; Ishii et al., 2006). Experiments conducted in surface water (Boehm et al., 2018; Bussi et al., 2017; Noble et al., 2004; Sokolova et al., 2012), sand (Staley et al., 2016) and soil (Ishii et al., 2006) suggest that fecal microbe persistence decreases with increasing temperature.

Predicted increases in water surface temperatures associated with climate change (IPCC, 2014; IPCC, 2018) can directly affect growth and survival of microbes in waterways (Liu et al., 2006). The persistence of fecal microbes in the environment may decrease in response to predicted impacts from many climate change, since fecal microbes generally degrade faster when temperature increases (i.e., bacterial die-off processes are enhanced; Boehm et al., 2018; Bussi et al., 2017; Noble et al., 2004; Sokolova et al., 2012). For example, there is a reported negative association between *Campylobacter* and temperature (Hokajarvi et al., 2013; Viau et al., 2011). Alternatively, rising temperatures may increase the occurrence of some pathogenic microbes. The presence of *Salmonella* spp. in Hawaiian coastal streams was positively correlated with water temperature (Viau et al., 2011). Likewise, the presence and persistence of *Vibrio* spp., which are capable of multiplying in water environments, are closely and positively correlated with water temperatures (Baker-Austin et al, 2016; Baker-Austin et al., 2013; Huehn et al., 2014; Sterk et al., 2015).

Factors such as temperature, solar inactivation, nutrient availability, and community interactions can have substantial impacts on the survival of FIB, opportunists, and pathogens at the sand-water continuum. However, the effects of these factors are complex, and may not be generalizable across the many pathogens that may be present in sand, these knowledge gaps contribute a great measure of uncertainty to estimates and models of the effects of global change on human health risk from recreational exposure to beach environments.

6. Putting it all together: modeling approaches for FIB in the sand-water continuum

Mechanistic models aim to describe complex phenomena by simulating the dynamics of contributing components. Such models are useful tools for understanding the relative importance of various fecal sources, fate processes, and transport pathways for FIB and pathogens. Furthermore, they can make predictions about the sanitary quality of beach systems and their impacts on human health. A conceptual model summarizing FIB sources, fate and transport processes in the beach system described in previous sections is shown in Fig. 3.

Existing process-based models of FIB in surface waters use some form of the advection-dispersion-reaction equation (shown below in its unsteady, 3D form):

$$\frac{\partial C}{\partial t}+u\frac{\partial C}{\partial x}+v\frac{\partial C}{\partial y}+w\frac{\partial C}{\partial z}=\frac{\partial}{\partial x}\left(K_H\frac{\partial C}{\partial x}\right)+\frac{\partial}{\partial y}\left(K_H\frac{\partial C}{\partial y}\right)+\frac{\partial}{\partial z}\left(K_V\frac{\partial C}{\partial z}\right)-S \quad (2)$$

where (u, v, w) are the unsteady, 3D velocity components in the coordinate directions (x, y, z) respectively (z denotes the vertical coordinate and t is time), C denotes the FIB concentration, $(K_H K_V)$ are the horizontal and vertical mixing coefficients and S is a general loss term ($S = kC$ if a first order removal rate constant k is used). Hipsey et al. (2008) provided a general formulation for the net loss term that integrates growth, water temperature, salinity, dissolved organic carbon, base mortality, light-mediated inactivation, pH, dissolved oxygen, settling losses, suspended sediment concentration, and predation and grazing losses. However, a variety of simpler net loss terms have been used successfully in localized marine and freshwater environments (Gao et al., 2015; Safaie et al., 2016). A detailed review of the FIB fate and transport processes in surface water and their mathematical representation is available in Nevers et al. (2011), though more effective incorporation of sand microorganisms and interactions into models is likely to improve model predictive ability.

Improved modeling of interactions between FIB and sand/sediment reservoirs may be key to further improving the performance of the current generation of hydrodynamic surface water models. Recent work on modeling FIB transport within sand at marine and freshwater beaches includes considering sediment

transport and sediment-bound FIB transport (Brown and Boehm, 2016; Feng et al., 2015; Gao et al., 2011; Thupaki et al., 2013) within the beach and in nearshore waters.

Different approaches have been used to model interactions between FIB and particles in the past, including: (a) models of attachment–detachment kinetics based on the mobile–immobile framework used in subsurface transport modeling (Brown and Boehm, 2016; Vangenuchten and Wagenet, 1989) and (b) sorption isotherm-based approaches typically used to describe partitioning of chemicals in the environment (Gao et al, 2011, 2015; Thupaki et al., 2013). Models such as those from Gao et al. (2011) use a linear coefficient $K_D = (C_S/C_D)$ for sediment-water partitioning of enterococci based on the assumption that attachment–detachment dynamics are "fast" relative to the time scales associated with advection and dispersion. Here C_S, C_D denote the mass-specific (e.g., CFU/g) concentration of attached FIB and volume-specific (e.g., CFU/mL) concentration of unattached FIB, respectively. While the use of a partition coefficient may approximate the distribution of FIB empirically in some locations, there is limited evidence that suspended and sorbed FIB are in equilibrium or that a single partition coefficient is generalizable for all environments (Nevers et al., 2011).

An alternate approach modeling dynamic FIB–particle interactions is to quantify sediment (and associated FIB) transport as discrete groups based on particle size – for example, relatively "fine" particle classes with which *E. coli* are known to readily associate (Brown et al., 2013; Hipsey et al., 2006; Jeng et al., 2005; Krometis et al., 2007) and a "coarse" size class – to simulate changes in bed morphology and the release of FIB from the sand reservoir. Rules for exchange of FIB between the water column and the bed sediment must be defined. For example, to quantify sediment-related enterococci at Hobie Beach in Florida, Feng et al. (2013) modeled "clean" and "contaminated" sand classes and their changes through time. The distribution of the contaminated sand fraction in this approach describes the availability of FIB for resuspension. In contrast, the models described in Gao et al. (2011) and Thupaki et al. (2013) used a single particle size class to successfully improve upon models without sediment contributions.

If FIB models considering sediment-bacteria interactions are extended to include multiple size classes (e.g., fine and coarse sediment), model complexity increases significantly, introducing a large number of parameters and assumptions that are difficult to constrain and justify. Therefore, questions of model parsimony, complexity and equifinality (Beven, 2006) should be addressed based on a careful comparison of different approaches.

In addition, novel field experiments and data that elucidate fundamental mechanisms of sediment transport with a focus on FIB transport and biofilms are needed. For example the model of biofilm dynamics presented in section 3 and in Vignaga et al. (2013) suggest that biofilm on sediments behaves like an elastic membrane that ruptures catastrophically above a velocity threshold. Current sediment transport formulations (Chao et al., 2008; Gao et al., 2011) assume that grains of sand roll over one another at some critical shear stress and do not consider catastrophic biofilm rupturing. The inclusion of biofilm dynamics in process-based models of FIB in surface waters and at the sediment-water interface may improve model predictions.

Accurate simulation of processes at the sand-water continuum, such as wave run-up on the beach face, cyclical wetting and drying associated with tides and waves, infiltration of water and lateral movement of water and FIB close to the sand-water continuum, all require high spatial resolution (cm to meter scale). Additionally, an understanding of how temporal changes associated with climate change and anthropogenic effects impact these processes is also key to modeling them. However, it is unclear how representative available FIB monitoring data and model approaches are for small-scale processes in beach systems, or conditions predicted by climate change scenarios (Alexander et al., 2006; Brown et al., 2008; Sneed, 2017). For example, the "fast" FIB attachment–detachment kinetics assumption that justifies the use of a linear partition coefficient is based on groundwater studies, as discussed in Bai and Lung (2005). Data from streams and agricultural soils suggest the opposite generalization, i.e. "slow" kinetics relative to advection and dispersion based on evidence that bacteria, once attached, will remain in that state as part of a biofilm (Jamieson et al., 2005). Additional field data from marine and freshwater sites collected under various hydrometeorological conditions will help test and validate new model frameworks for the effective simulation and prediction of nearshore FIB fate and transport at the sand-water continuum.

Modeling sediment- and sand-bound FIB transport is a complex, but important step in understanding beach microbial dynamics. Subsurface microbial transport and interactions at the sand-water continuum remain critical knowledge gaps, especially in turbulent and wave-impacted environments. The advection-dispersion-reaction equation serves as the foundation for these models, but relevant parameters, boundary and initial conditions, and model complexity can vary greatly, depending on the focal nearshore environment and localized factors. Existing models are at odds in terms of how to predict dynamics at the sand-water continuum, especially in light of climate change effects. Additional field data validation and model development focused on sand- and sediment- FIB transport effects on beaches will be crucial to the improvement of nearshore water quality simulations and prediction of future climate change effects.

7. Emerging concerns and challenges in the face of predicted climate change

As highlighted throughout this review, any advancements in modeling or managing the sand-water continuum with regard to beach water quality must be responsive to the impacts of climate change on microbiological contamination at recreational beaches. As the water and land environments warm and experience changes in storm frequencies, the sand-water continuum will likely experience changes in both the abundance and diversity of FIB and pathogens potentially persisting in these habitats. Such changes may present unique and unprecedented challenges for recreational water quality management.

7.1. Increased storm frequency

Coastal areas are expected to experience increases in the frequency and intensity of storm events as a result of climate change (IPCC, 2014). As these areas begin to experience more frequent and intense storm events, localized flooding, runoff and combined sewer overflow events will also become more frequent, making these sources of contamination to both the beach water and the sand-water continuum more commonplace (Kessler, 2011; Roca et al., 2019; Trtanj et al., 2016; USEPA, 2008). Contaminant releases from wastewater treatment plants, sewage systems and industrial contamination sources will increase by 50–120% in the US Great Lakes region (Patz et al., 2008), while groundwater contamination via the infiltration of microbes through the vadose zone is expected to impact well water quality more frequently (Arnaud et al., 2015; Klove et al., 2014). Additionally, predicted changes in mean sea levels across latitudes suggest that global average coastal water levels 0.26–0.85 m higher than observed in 1986–2005 will impact beaches in tropical and temperate areas by 2100 (IPCC, 2014). Increased water levels, combined with predicted changes

to storm frequency and wind patterns, will lead to larger areas along the marine shoreline affected by wave and tidal action and periodic wetting, enhancing the exchange of microbial contamination between the sand and water.

7.2. Warming waters

In similar fashion to the range shifts seen in terrestrial and aquatic macrofauna (Kerr et al., 2015; Parmesan and Yohe, 2003; Perry et al., 2005), it may be expected that microorganism community ranges will shift poleward in response to climate change (Burge et al., 2014; Ford, 1996; Lafferty, 2009; Trtanj et al., 2016). As global water (Fig. S3) and air temperatures are predicted to warm, the thermotolerance thresholds of some microorganisms may be exceeded, while the warmer conditions may lead to increased decay rates and die-offs for some species (e.g. FIB; Ishii et al., 2006). This may open up niches in temperate coastal areas for more thermotolerant microbes that had previously been found in lower latitudes, such as *Vibrio parahaemolyticus, Vibrio vulnificus,* and *Naegleria fowleri* (Baker-Austin et al., 2013; Kemble et al., 2012; Martinez-Urtaza et al., 2010). Additionally, pathogens, opportunists and FIB may be transported poleward directly via hurricanes, cyclones, and tropical storms, known products of ocean surface temperature changes (Wu et al., 2016).

Elevated water and air temperatures may also foster the growth of antimicrobial resistant microorganisms (MacFadden et al., 2018). These microorganisms, unlike most FIB, can be directly associated with adverse human health outcomes (Cosgrove and Carmeli, 2003; de Kraker et al., 2011; O'Gara, 2017). Additionally, the expansion of previously undetected microorganisms to temperate regions, combined with possible increases in antimicrobial resistance and the propensity of microbes to form biofilms within the sand-water continuum suggests that the risks to beachgoers from new and dangerous pathogens will increase in response to climate change.

7.3. Drought impacts

While much attention has been devoted to increases in storms as a result of climate change, the effects of drought will also have an impact on recreational water and sand quality, though additional research is required to determine whether the effects are positive or negative. Drought conditions can limit the runoff flowing to beaches from upstream in the watershed, leading to declines in runoff-associated contamination and potentially higher recreational water quality (Vu, 2018). Conversely, declines in water levels due to drought will likely expose more of the beach face, potentially leading to changes in biofilm development and microbial community growth within the sand-water continuum. Smaller beach face areas subject to wave- and tide-associated periodic wetting due to water level declines may be expected to facilitate biofilm growth and reduce areas prone to biofilm detachment (Boehm and Weisberg, 2005; Phillips et al., 2014). However, a lack of moisture sources to mediate temperature in the sand may result in enhanced decay of microorganisms (Ishii et al., 2006; Mika et al., 2009). Lower water levels will also yield increased surface area for which beachgoers to contact the sand-water continuum and its associated microbial communities, potentially increasing the risks to beachgoers connected to microbes in the sand-water continuum. Beach management for public health will, thus, require adaptation for increased beach usage associated with greater surface area for beachgoers to utilize as well as changes to biofilm dynamics and resulting risks to beachgoers.

8. Management for the future

The predicted effects of climate change necessitate a paradigm shift within recreational beach management and policy development (Coffey et al., 2014). Effective beach management to protect public health into the future will not only account for contamination in the sand-water continuum but will also take an adaptive approach that can be adjusted in response to the impacts of changing storm frequency, flooding effects, and upstream contamination potential. Beach managers, particularly in temperate regions affected by warming air and water temperatures, will need to monitor both beach sand and recreational water for a greater variety of pathogenic species than they have in the past, to ensure public health.

Predicted droughts may also necessitate more effective management of recreational water quality, especially terms of the exchange of water and associated microbes between sand and water environments at the beach. This will require an adaptive and holistic management strategy. An adaptive approach that can be altered in response to climate change impacts will be most effective for management of water resources under rapidly and substantially changing conditions. Likewise, a holistic, “beachscape” approach to management of recreational water systems can effectively account for the entire beach system (Weiskerger and Whitman, 2018). Such an approach will incorporate water, sand, wildlife, human, and upstream contamination/runoff impacts on beach health, rather than simply focusing on recreational water (Kelly et al., 2018), and can lead to management and policy that preserves human, wildlife, and environmental health at beaches.

9. Conclusions

- Beach ecosystems are dynamic mosaics of aquatic, sand, and sediment habitats.
- The sand-water continuum plays an important role in the accumulation, transport, and persistence of microbial contaminants at beach systems, though it is difficult to model and is often overlooked in nearshore research, management, and policy-making efforts.
- Local hydrometeorological and infrastructural conditions can influence contamination patterns at beaches over time and across space, so localized monitoring and modeling efforts are needed to effectively manage beach systems in the face of contamination in both the nearshore and at the sand-water continuum.
- Beach management policies are lagging behind the research into the role of sand as a contamination source; however, several nations and the WHO have recognized sand as an important reservoir of FIB and route of exposure to pathogens.
- The recognition and ongoing discussion of sand as a contamination source at beaches may signal a paradigm shift toward more holistic, “beachscape” frameworks for guiding beach research and management actions.
- Future efforts to model nearshore human health risks must be robust and adaptable to account for the association/disassociation of human health relevant biofilm- and particle-bound constituents associated with beach sands and underlying sediments, as well as uncertainty associated with changing weather and climatic patterns.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.07.006.

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

All credited authors participated in the development and organization of the ideas herein, as well as writing and editing of this review. Section leaders included Valerie J. Harwood and Michael J. Sadowsky (Getting Our Feet Wet), Thomas A. Edge and Erin M. Symonds (Pollution Pathways), João Brandão and Helena M. Solo-Gabriele (Hangouts), Clare Robinson and Laura J. Vogel (Cruising), Gregory T. Kleinheinz (Bummers), Alexandria B. Boehm and Mantha S. Phanikumar (Putting It All Together), Christopher D. Heaney and Tarja Pitkänen (Climate Change), and Chelsea J. Weiskerger and Julie L. Kinzelman (Emerging Concerns and Challenges, and Conclusions). João Brandão and Chelsea J. Weiskerger led the effort to develop and build the paper. Beyond section leaders, remaining authors contributed to sections throughout the paper and provided editing assistance. Valerie J. Harwood took the lead on reviewing and editing the final draft assisted by Michael J. Sadowsky, Julie L. Kinzelman, Warish Ahmed and Brian Badgley.

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Supplementary Material:

Impacts of a Changing Earth on Microbial Dynamics and Human Health Risks in the Continuum between Beach Water and Sand

Contents:

Table S1: Representative waterborne pathogens and respective estimated infective dose (not-specifically waterborne estimations)

Type	Species	Disease/illness	Infective dose	Reference
Virus	Norovirus	Diarrhea	~20 viral particles	(Hall, 2012)
Virus	Adenovirus	Diarrhea and respiratory infection	~150 PFU	(Canada, 2002)
Virus	Enterovirus	Diarrhea, vomiting, fever, skin rash, conjunctivitis	<18 PFU	(Canada, 2001)
Virus	Rotavirus	Diarrhea, vomiting, fever	1 PFU	(Graham et al., 1987)
Bacterium	*Campylobacter jejuni*	Diarrhea	800-10^6 CFU	(Black et al., 1988)
Bacterium	*Escherichia coli* O157:H7	Diarrhea, kidney failure	1-100 CFU	(Paton and Paton, 1998)
Bacterium	Toxigenic *Vibrio cholerae*	Cholera	10^3-10^8 CFU	(Schmid-Hempel and Frank, 2007)
Bacterium	*Salmonella enterica*	Diarrhea, fever, abdominal pain	>10^5 CFU	(Kothary and Babu, 2001)
Helminth	*Ascaris lumbricoides*, *Trichuris trichiura*, *Nector americanus* and *Ancyclostoma duodenale*	Diarrhea, abdominal pain, malnutrition	~10 larvae	(WHO, 2006)
Protozoan	*Giardia lamblia*	Diarrhea, abdominal pain, nausea	10-100 cysts	(Leggett et al., 2012)
Protozoan	*Cryptosporidium parvum*	Diarrhea, abdominal pain	1-5 oocysts	(Guerrant, 1997)

Fungi	*Aspergillus* spp.	*Aspergiloma, Aspergilosis, Onycomycosis,* Allergy	*Not available*	(Lee et al., 2016; Sabino et al., 2014)
Fungi	*Candida albicans*	*Candidosis (systemic and localized)*	*Not available*	(Loureiro et al., 2005; Vogel et al., 2007; WHO, 2003)

CFU: colony forming unit; PFU: plaque forming unit.

Figure S1: Biofilms found on sediments of fluvial beaches. In panel A, a lower magnification of the sediment is shown. The red arrow highlights the presence of a bacterial biofilm. The blue rectangle marks the area shown in more detail in panel A.1. The bacteria (Aeromonas sobria and Enterobacter cloacae shown by red arrows) are surrounded by a thick EPS layer highlighted by blue arrows. Panel B shows the close interaction between phytoplankton organisms and bacterial biofilms, (highlighted inside the red rectangle), where adherent bacteria could be observed on the phytoplankton filament (Jordao, 2016).

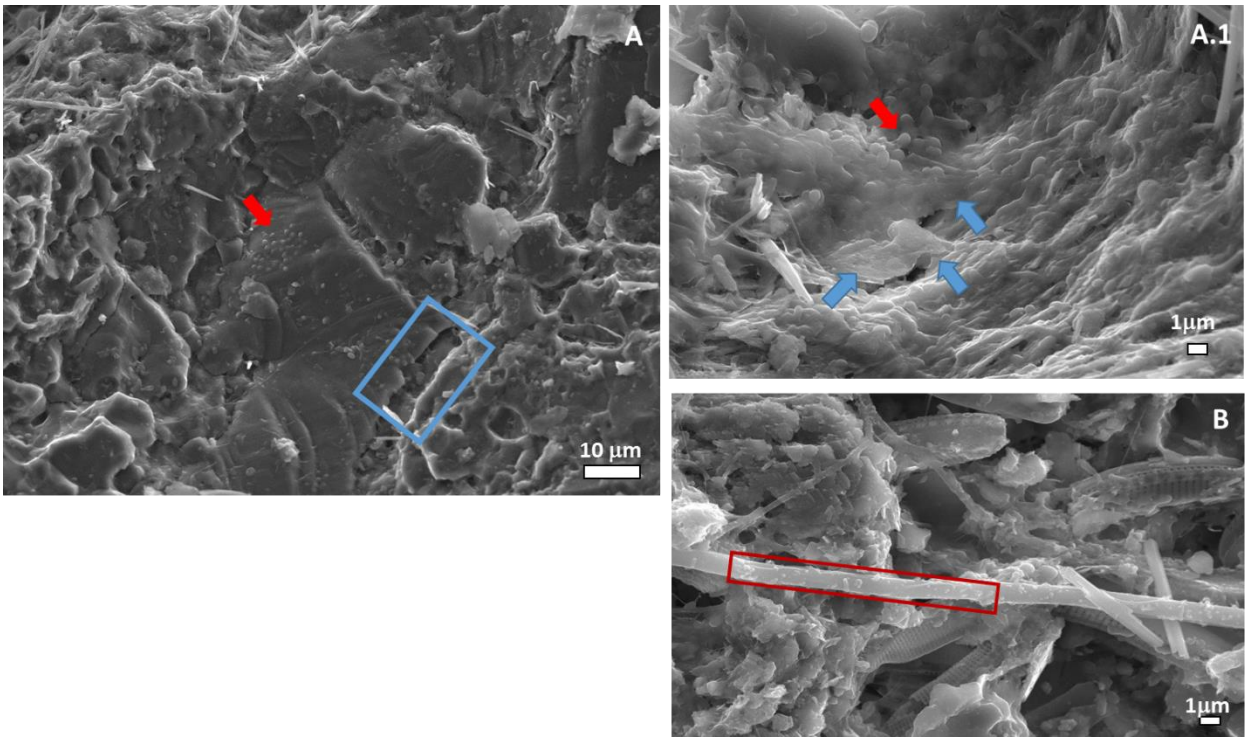


Figure S2: Bathymetry and EPS levels at beaches in south Florida, U.S. Dotted line represents the 6 m contour for water depth. The bathymetry illustrates the gentler bottom slopes at Crandon and Bill Baggs Beaches relative to beaches to the north (Piggot et al., 2012).

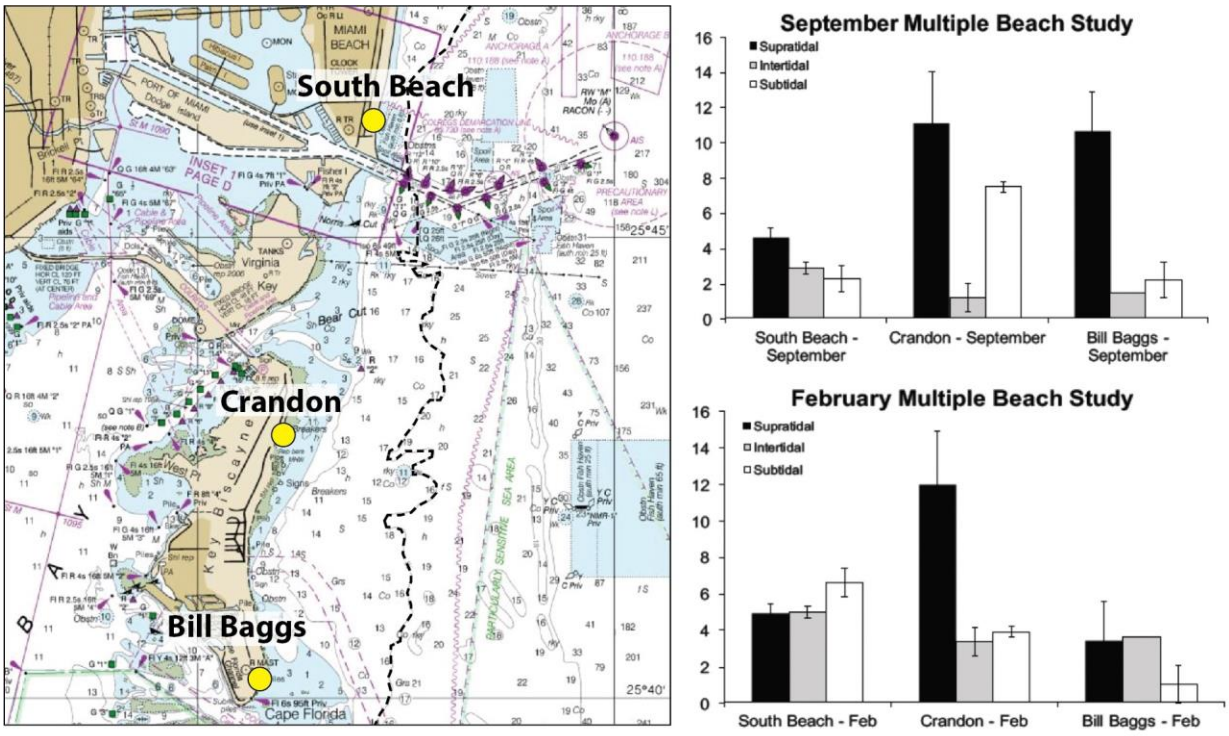
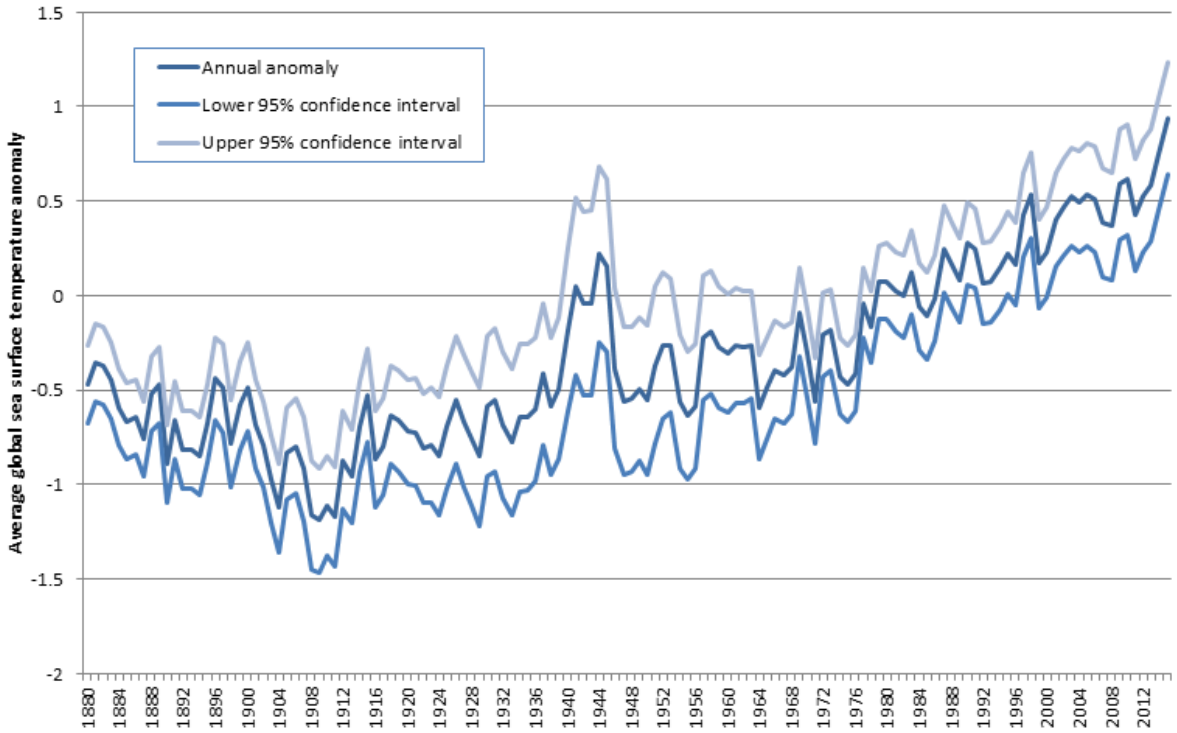


Figure S3: Annual anomaly in average global sea surface temperature in 1880–2015 (NOAA, 2016).



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Chapter 3

Microorganisms in Beach Sand: What Do We Still Not Know?

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Microorganisms in Beach Sand: What Do We Still Not Know?

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Sampling and Safety Parameters

Beach sand is a known reservoir of pathogenic and opportunistic microorganisms as well as of fecal indicator organisms, which are used to establish the safety of recreational water. Many aspects on beach sand microbiota have been addressed before in this encyclopedia: survival of microbes, predation, management, and most importantly, the possible health implications of exposure when one lies down on a beach (in Efstratiou, 2018 "Microorganisms in Beach Sand: Health Implications"). However, one of the issues with sand analysis that is often mentioned but seems to have no obvious solution is that sand is patchy and lacks, therefore, a gradient or steady distribution of any parameters used to assess its safety for recreational use. Unlike water, for which sampling is generally expected to represent a large volume, when sampling sand, the question "is my sample representative of this beach?" is inevitably answered as … "no!." No matter how many portions one collects to create a composite sample (incremented sampling), or how many batches are collected and analyzed individually, one can never expect all of the length and all the width of a beach to be represented in that manner. In fact, not even the immediate vicinity of a sample site will certifiably be reflected in it. A simple deposit of excretions by a warm-blooded animal will leave a small area of sand loaded with fecal organisms of animal origin. And these fecal organisms may still be found long after the fecal matter has disappeared from sight. Additionally, a whole step-by-step series of dominating flora (from bacteria and yeast to mold and even other more complex life forms) will ensure the recycling of all of the organic matter, and may thus be detected as well.

Yet, limiting as the sand patchiness of microbial load and variety may seem, the relevance of sand analysis should not be minimized; analytical procedures will always have limitations. In this case, historical data takes up a relevant role, permitting the detection of mean values and outliers of normal behavior, muffling the relevance of loose analytical events. Sand analysists must pursue this target if the work is to be used for effective health protection purposes. A combination of incremental sampling and previous knowledge of hotspots of microbial contamination (worst-case scenario) should help make sampling both relevant and representative for any beach.

Intrinsic Versus Extrinsic Microbiota

Sampling is not the only limitation to microbial sand analysis. Microbiota is intrinsically associated with geographical regions, due to local life, climate, geological features, etc. This means that the "one size fits all" solution, in terms or future regulation, is simply not possible. The results of one site mean nothing in another with different characteristics, even if nearby. A risk-based approach combined with historical data is the solution. By means of this approach, the intrinsic flora of a beach is complemented by species brought in by its visitors in their skin and other parts of the body.

The current water safety parameters (fecal indicator bacteria) are very present in sand microbial safety literature, which substantiates their relevance for sand also. They serve the purpose of evaluating sand as a diffuse pollution source for the nearing water, and to provide a simple and well-established indication of fecal contamination of the sand. What still needs to be agreed upon, concerning parameters, preferably based on risk assessment (local/regional level) or epidemiological data, are which other bacteria (of non-fecal origin), fungi and parasites should be looked at in order to assess the safety of beach users. The potential for causing infection is not easy to determine. It is very much dependent on the host, both intrinsically and behaviorally. Considering fungi, for example, there are pathogenic species but most fungi are in fact opportunists. It is estimated that about 25% of the world population suffers from superficial fungal infections, most of which are *Tineae* and onychomycosis. The majority of these two pathologies are caused by dermatophytes, generally accepted as pathogenic within the Medical Mycology community. Finding them in sand reveals the potential for infections. The recovery of fungi from sand through extraction with water in orbital shaking has been estimated to deliver very low yields (nearing 1%). This means for each colony found in sand there were 99 that went undetected. The relevance of allergenic and opportunistic fungi remains a mystery but will inevitably be related to the fungal load and susceptibility/reactivity of a host.

A few non-enteric bacteria have been identified as of interest in this context, due to previous reports of their findings in beach sand, namely *Pseudomonas aeruginosa*, *Vibrio* spp., and *Staphylococcus aureus*. *Vibrio* is currently being monitored with a predictive tool at the European Center for Disease Control and Prevention (ECDC) in water since cases of necrotizing fasciitis caused by *V. cholerae* were reported in Europe in 2016. Protozoa identified as relevant for sand and not intrinsic inhabitants of beach sand, are species of the genera *Giardia* and *Cryptosporidium*. Endemic fungi of the genera *Histoplasma*, *Blastomyces*, *Coccidiodes*, and *Paracoccidiodes*, and some non-endemic mentioned in "**Risk Quantification**" section, represent some of the more serious pathologies both in immunocompromised and in immune-competent patients in the Americas. Helminths should also be considered in regions following the risk-based approach. Regarding viruses, genetical material of gastrointestinal (GI) viruses has been found in beach sand but viability studies are still the only tool available to identify risk, based on the presence of the actual viable (viral) pathogen. **Table 1** lists various pathogens and opportunistic microorganisms of interest in this context.

Table 1 Microorganisms identified as of interest for sand analysis and health protection of beach users and list of respective symptoms and pathologies (GI stands for gastrointestinal)

Type	*Species*	*Disease/illness*
Virus	Norovirus	GI infection
Virus	Adenovirus	GI and respiratory infection
Virus	Enterovirus	GI infection
Virus	Rotavirus	GI infection
Bacterium	*Campylobacter jejuni*	GI infection
Bacterium	*Escherichia coli* O157:H7	GI infection
Bacterium	*Vibrio cholerae*	GI infection
Bacterium	*Salmonella enterica*	GI infection
Bacterium	*Pseudomonas aeruginosa*	Otitis, respiratory, superficial, genito-urinary and GI infections
Bacterium	*Shigella spp*	GI infection
Bacterium	*Staphylococcus aureus*	Skin, invasive/systemic infection, endocarditis, skeptic arthritis
Helminth	*Ascaris lumbricoides*, *Trichuris trichiura*, *Nector americanus* and *Ancyclostoma duodenale*	GI infestation
Protozoan	*Giardia lamblia*	GI infestation
Protozoan	*Cryptosporidium parvum*	GI infestation
Fungi	*Aspergillus* spp.	*Aspergiloma, Aspergilosis, Onycomycosis,* Allergy
Fungus	*Candida albicans*	*Candidosis* (systemic and localized)
Fungi	Endemic Fungi (*Histoplasma capsulatum, Blastomyces* spp., *Coccidiodes* and *Paracoccidiodes*)	*Histoplasmosis, Coccidiomicosis, Blastomycosis, paracoccidioidomycosis*
Fungi	*Cryptococcus* spp.	Cryptococcosis
Fungus	*Cladophialophora bantiana*	Phaeohyphomycosis
Fungi	Keratinophilic fungi	*Tineae*, onychomycosis and Fusariosis

Risk Quantification

There is a huge void in data for most beaches but also a lack of reasoning for the interpretation of analytical results when considering public health. One link has been established between sand analysis and influence to the health of beachgoers: an epidemiological study that was carried out in North America and helped demonstrate that there is an increase in GI illness of a population that plays with sand when compared with another that does not. This relation does not consider the causative agents but rather the symptoms resulting from the direct exposure.

Exposure to fungi in any environmental setting is of interest, as it is estimated that about 90% of all fungal infections in humans originate in the environment. But, an epidemiological assessment of any pathology derived from fungal exposure at the beach (that excludes other possible sources of exposure) still needs to happen. Only this way will it be possible to demonstrate the need to look into fungal contaminants at a beach. Quantitative Microbial Risk Assessment could be an alternative but it's difficult to generate estimates, due to the opportunistic nature of most fungi and to the fact that there are several possible unrelated ailments caused by a single species of fungi. A good example of this is *Aspergillus* spp. which can cause asthmatic flair-ups but also invasive fungal infections in susceptible individuals with concomitant compatible pathologies. Fungal ailments thus derive very much from the individual characteristics of the host (patient), especially if suffering from a debilitating condition.

A special concern with fungal contaminants has been the biosecurity level three (BSL3) neurotropic fungus *Cladophialophora bantiana*. Black mold typically thrives in environments rich in hydrocarbons with all kinds of radiation, and beaches are thus traditional niches due to some degree of contamination by fuel originating in boats and aquatic motorbikes. At first glance, *Cladophialophora* spp. can easily be confused with *Cladosporium* spp. The colors and textures of their colonies do not differ very obviously. A good approach is to incubate samples at 37°C where *Cladosporium* spp. is unable to grow. This will help differentiate any fungus that has the potential to infect warm-blooded animals. The same concern with increased pathogenic potential occurs at urban riverbanks that are heavily visited by pigeons, known carriers of *Cryptococcus* spp. Both *Cryptococcus* spp. and *Cladophialophora bantiana* display neurotropic behavior, which makes them very serious threats to the patients they infect.

Antimicrobial Resistance

Lastly, another issue that needs to be taken into account in terms of beach exposure to fungi and bacteria, is the serious rise in antimicrobial resistance (AMR). Resistance genes are spreading in the environment (by means of selective pressure) and any setting must be seen as a possible reservoir to a probable infectious complication. In fact, in many countries, there is hardly any blockage towards waterborne pathogens ending up in recreational waters and sand. Europe has done a lot of work in this field during the

implementation of the revised bathing water directive (BWD) up till 2015, and that tightened the thresholds of fecal indicator parameters. Yet, despite all the BWD implementation actions, studies have recently confirmed the emerging of third-generation cephalosporin resistant Escherichia coli in coastal recreational waters of the UK, which probably originated in land. In fungi, there are *Candida* species that cause superficial infections and that are intrinsically resistant to azoles, whilst others exhibit some resistance to Fluconazole, one of the main therapeutic options as systemic anti-fungal therapy (also for superficial infections). Azole resistance that is thought to have epicentered in Western Central Europe is spreading fast within some species of *Aspergillus* section *fumigate*.

Global Warming is a Game Changer

As global warming takes place, and the human population continues to grow, coastal areas around the world are expected to suffer the stress associated with all kinds of human activities. Water is essential for living and humans always showed a tendency to congregate near water sources for several reasons, fishing and bathing above all. This is especially relevant when considering the expected desertification of certain regions, like the South of Europe. A steady increase in the consumption of drinking water and wastewater production in waterfront communities is thus likely to take place. Lack of drinking water is a strong possibility and a well-established link to human disease and two additional risks concern wastewater facility management: firstly, when there is an excess of wastewater facilities tend to collapse and direct discharges in water masses take place. Secondly, the excess population in waterfronts and the resulting increase of potential environment contaminating facilities, like sewage treatment plants, increase the risk of destruction during severe storms, already common in certain regions of the globe. Since these storms are expected to increase in frequency and intensity, this problem might become more and more relevant. Besides, sand microbial contamination can come from the land via run-offs and direct deposition by all kinds of living creatures, as mentioned above. Heavy rain and severe storms will contribute to the displacement of microbes and resulting alteration in the sand communities composition. Hurricanes have the potential to pick up microbial life from one place and release it back in another, maybe hundreds of miles away; where growing conditions may permit settling and hence alter the local microbiome by that reason also. This may change the local clinical paradigms associated with any region of the globe.

Summarizing: Sand microbial contaminants need to be addressed in regulation within a relatively short period of time, in order to allow some planning based on a knowledge that still needs to be built.

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Chapter 4

Fungal Exposure and Relevant Recreational Settings

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Fungal Exposure and Relevant Recreational Settings

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Fungi and Recreational Water Environments

Recreational water environments are often where we wear less protective clothing against environmental factors, like when we are at the beach. Possible activities that can foster exposure to fungi in recreational waters' sites and settings include the following:

(1) Bathing in recreational and medicinal waters (public swimming pools, beaches, saunas, spas, etc.)
(2) Sunbathing
(3) Water sports (rowboat, kayaking, surfing, fishing, diving, etc.)

Exposure

Swimming Pools

Fungal infection can be a substantial health concern at public swimming pools, due to the prevalence of fungi on hard surfaces, mainly those partly submerged in water, as published by Ekowati *et al.* (2018). This author postulated that dermatophytes tend to follow the trajectory of the users, from the pool, to the dressing and shower areas, aided by cleaning instruments. These types of fungal contamination are present mainly on solid surfaces in swimming-pool environments. The recognition of public swimming pools as a high risk for *tinea pedis* and onychomycosis is not a new subject. The first time dermatophytes were successfully isolated from shower floors was in 1947 but they still remain an issue today. This is not surprising, though, if we consider that worldwide, 25% of the population suffers from a superficial fungal infection of some kind. Besides the fungi listed above, the yeasts *Candida* and *Rhodotorula* are frequently encountered in water in swimming pools (Brandi *et al.*, 2007; Jankowski *et al.*, 2017).

Water-borne fungi often thrive in recreational facilities, such as pools and especially high humidity saunas. These can include black yeasts and yeast-like *Aureobasidium*, *Cladophialophora*, *Cyphellophora*, *Exophiala*, *Phialophora*, *Phoma*, and *Ochroconis* (Matos *et al.*, 2002; Hamada and Abe, 2009; Novak Babič *et al.*, 2018). The damp, warm environment and necessary nutrients for the growth of black fungi make them a probable nuisance for facility management, but above all a potential problem for the health of the visitors and users. Some of the black fungi are considered opportunistic pathogens, causing superficial infections and in some cases of trauma, invasive infections, mainly in form of mycetoma, but occasionally as a disseminated (systemic) infection. Although the most commonly reported health issues related to leisure activities in swimming facilities are on average caused by dermatophytes, other fungi present in high concentrations may affect well-being by triggering an allergic response in susceptible individuals. Simon-Nobbe *et al.* (2008) describe *Alternaria*, *Aspergillus*, *Bipolaris*, *Cladosporium*, *Curvularia*, and *Penicillium* as triggers of rhinitis; *Alternaria*, *Aspergillus*, *Cladosporium*, *Helminthosporium*, *Epicoccum*, *Aureobasidium*, and *Penicillium* as triggers of allergic asthma; and *Malassezia*, *Sccharomyces cerevisiae*, *Alternaria*, *Candida*, and *Trichophyton* as triggers of atopic dermatitis.

Other health implications, such as intoxication due to mycotoxins, sinusitis, asthma, and infections of the respiratory tract, are not likely to occur in occasional visitors but should be taken into consideration for workers in such facilities. Amongst the most problematic molds on wet concrete and finishing materials on ceilings indoors is the presence of *Stachybotrys* spp. Prolonged exposure to toxic metabolites by these fungi can lead to respiratory infections, irritation, and allergies (Novak Babič *et al.*, 2018). Fungal presence in water and on surfaces is nonetheless difficult to control due to fungal resistance to most agents that can be used for their removal, namely bleach and UV radiation (DEFRA, 2011; Novak Babič *et al.*, 2018).

While dermatophytes, yeasts and yeast-like fungi dominate lower parts of wet surfaces where exposure happens through skin, upper ceilings often promote growth of filamentous fungi transferred by air with possible effects on our respiratory system. Viegas *et al.* (2011) found no association between air and surface contaminants, either in quality (species), or quantity (number of colony-forming units (CFU) per sample volume or area, respectively). In this study, the majority of the air contaminants in 200 L of air samples were *Cladosporia* (36.6%), *Penicillia* (19.0%), and *Aspergilli* (10.2%), whereas in surfaces, *Fusaria* showed the strongest presence, with 19.1% of positive samples. *Fusaria* is in fact a species complex that tends to prefer warmer but not dry climates, soil, and plants, as reported by many authors, but also shows up in aquatic habitats (Lamprecht *et al.*, 2011). Dominant species of this genus may vary between fresh water and seawater (Palmero *et al.*, 2009). The relevance in aquatic habitats of *Fusarium* species is their association with keratitis in patients with lesions after exposure to water. Contact lenses are also a risk factor for this pathology (Ahearn *et al.*, 2008). *Fusarium* is, however, not the only fungus able to cause keratitis. Khater *et al.* (2014) studied 66,303 cases of microbial keratitis and determined that 43.3% of the cases were of fungal origin (mostly *Aspergillus*), although in this study, the patients were mainly adults aged between 40 and 60 years, who had close contact with soil and outdoor sewage.

Sunbathing

Sunbathing is an activity that does not necessarily imply direct contact with recreational waters. By the coast, inevitably, people tend to get their daily dose of vitamin D production and energy at the beach. Considering that most beaches at the coast are composed of sand, which is where recreational beach users tend to spend most of their visiting time, one must wonder why has the regulatory community only worried about water as a potential source of disease. In fact, sand can harbor all kinds of pathogens and even other health threats (e.g. glass fragments and hematophagic flies, some of which are vectors of zoonoses).

Currently, the bathing water regulation is based on the World Health Organization's "Guidelines for safe recreational water environments", published in 2003 (WHO – World Health Organization, 2003). These guidelines recommend a limit of 200 enterococci to accept a 5% risk of developing an ailment associated with human fecal pollution during bathing. While fungi are not mentioned *per se* in the guidelines, they may be included in the equation to determine 5% risk of ailment. On the other hand, management of sand contamination is not included in the guidelines or subsequent regulations, even implicitly. The concept of sand contaminants is pointed out in chapter 6 of the guidelines (where even fungi are addressed) but without any actual conclusions or recommendations of parameters and respective levels. There has been a considerable amount of work published with results and discussions on the raison d'être of looking at contaminants in sand. In September 2014, a meeting in Lisbon, Portugal gathered professionals of several areas of expertise to discuss the most sensible approach for sand contaminant analyses, amongst other microbiological public health issues. Up for discussion were parameters, their limits, analytical methods and sampling representativeness as they relate to monitoring sand contamination. The group included bacteriologists, virologists, medical mycologists, and parasitologists both as scientists and technical staff, public health professionals, and beach managers. The outcome of that meeting was a white paper authored by a smaller group of participants who published the meeting's observations and recommendations (Solo-Gabriele *et al.*, 2016). **Table 1** lists those.

The group discussion focused on water environments but many of the recommendations are also valid for other sand environments, like sandboxes and recreational parks (see Section "Sandboxes"). The proposed structure for fungal analysis is a simple test based on undifferentiated colony count that can be performed in any water microbiology lab, unless there is reason to specifically identify or detect a species, like an overwhelming presence or outbreak. This requires a reference laboratory or at least an accredited procedure because the recent taxonomic revolution that is taking place in medical mycology, together with emerging anti-fungal resistance, makes the correct identification of fungi a detail of importance, especially during an outbreak.

Table 1 Observations and recommendations of the white paper addressing sand microbial contaminants

Observations

Beaches are a significant contributor to the pathogen load to which beach users are exposed.
At beaches not impacted by sewage, the source of pathogens originates from the local site and includes human visitors, animals, local runoff and the release of microbes from sand.
Studies have identified the presence of pathogens in sand and factors other than point source pollution that contribute to their presence (e.g. moisture, wrack, wildlife, domestic animals, beach morphology, currents).
Epidemiological studies have shown that children who play in sand are subject to higher rates of illness than those not playing in sand.
Measurements of fungi have not been included in beach epidemiological studies.
Considerable evidence exists that sand can serve as a reservoir of enteric microorganisms and fungi, which can be vehicles of disease transmission.
Highlight: Contaminated sands present health and economic costs that can and should be known by decision makers, communities and by individuals.

Recommendations

Identification of recreational beach areas.
Identification of disease-causing agent(s) (viruses, bacteria, fungi and protozoa).
Sampling strategy, control and monitoring programmes.
Methods that estimate risks from pathogens in the sand.
Assessment of exposure to microbes in the sand by contact, ingestion and inhalation.
Tools for identification of sources of microbes.
Evaluation of alternative fecal indicator organisms, to determine the presence of sewage and human waste.
Detection and quantification of microbe levels for specific pathogens with culture-based methods, q-PCR and beach metagenomes.
Determination of whether FIOs are indicative of fecal pollution that carries pathogens or if they have separated from their original source through survival and regrowth.
Development of regulatory standards that reflect microbial sources, pathogens they contain, and associated health risks.
Development of reliable sand collection methods since pathogen contamination at sandy beaches tends to be patchy.
Determination of beach sand quality at freshwater vs. marine beaches.
Assessment of beach sand quality based on contamination by land and air.
Creation of standardized methods to recover and disinfect pathogens from different types of sands.

Highlight: Available evidence should be evaluated by both scientists and regulators with a view to filling the data gaps, which should be followed by sound policy development for safeguarding public health.

Note: Adapted from Romão, D., Sabino, R., Veríssimo, C., *et al.*, 2015. Children and sand play: Screening of potential harmful microorganisms in sandboxes, parks, and beaches. Current Fungal Infection Reports 9, 155–163.

The relevance of sand as an exposure setting in beach and public health was confirmed by Heaney *et al.* (2012) with a controlled epidemiological study of 4,999 beach users and 144 wet sand samples tested for F+ coliphage, *Enterococcus*, *Bacteroidales*, fecal *Bacteroides*, and *Clostridium* spp. The study confirmed an increase in gastrointestinal illness associated with playing in sand on a beach. Although raising awareness to the confirmation of sand as an exposure vector at a beach, the study did not include other microbes or dry sand. Although fungi are known to be plated also directly from water, it is in the dry sand that most of the fungal contaminants can be found, despite the extreme temperatures and solar radiation to which they may be subjected. In fact, some of these fungi, as mentioned above, thrive on radiation. Finding black molds in beach sand seems to be a common situation (Romão *et al.*, 2015). Like other fungal groups, melanised fungi are brought to sand via different carriers – plants, animals, through anthropogenic influences including pollution, and water. However, they do differentiate from others in high concentrations of melanin in their cell walls and unique growth in thick meristematic clumps. Melanin has a protective role against irradiation, but also uses radiation to produce energy and thus helps fixate the micronutrients necessary for fungal development (Dadachova *et al.*, 2007; Dadachova and Casadevall, 2008). Thus, melanised fungi like *Trimmatostroma*, *Exophiala*, *Coniosporium*, *Phaeotheca*, and *Lichenothelia*, are often found on dry solid rocks, covered with salts and exposed to UV radiation (Sterflinger, 1998).

Extremophiles, such as the genera *Hortaea* and *Wallemia* have been isolated from salterns and are frequently present in ponds with high concentrations of salt. Prolonged exposure to those fungi was described as *tinea nigra*, where *Hortaea werneckii* grew on palms of salt harvesting workers. Occasional exposure can be related to spa treatments where mud from salterns is used. While *Hortaea* is widely present in seawater and sand, other black yeasts, such as *Cladophialophora*, *Aureobasidium*, and *Exophiala* likely occur more locally in association with the presence of long-chained hydrocarbons, namely oils and their derivates (Prenafeta-Boldú *et al.*, 2006; Isola *et al.*, 2013). Also, *Aureobasidium* and *Exophiala* at beaches in Greece have been associated with geochemical properties of sand (Efstratiou and Velegraki, 2009).

How relevant are fungi at the beach, in terms of exposure? Nobody knows. The most common dermatophytes and the most commonly found yeast in human infections, *Candida albicans*, do not seem to survive the heat of the sand at a sunny beach. They sometimes can be found in highly populated beaches, suggesting a recent contamination of anthropogenic origin, rather than resilience. This does not exclude the chance of a transfer between individuals before inactivation, nor does it exclude less sunny beaches that may foster longer survival times. More work is needed in order to assess the actual risk of fungal ailments of recreational beach users.

Also, if we want to consider looking into a possible outbreak, how do we know that the number of cases isolated from recreational beach users did not contaminate the sand, rather than being contaminated by the sand? Fungal outbreaks are usually slow and scattered, with low numbers of cases. They are not explosive like those associated with viruses and certain bacteria. Many patients may have been infected during their holidays and then go home before the onset of the symptoms, rendering the connection between a beach visit and infection unlikely to be found. Residents can be screened during a bathing season, but the local area includes many other possible indirect contamination surfaces/media besides beach sand. It is thus extremely difficult to find an outbreak, unless obvious in a geographically confined yet relevant number of cases. But there may be an alternative to detection, based on modeling of expectations and Quantitative Microbial Risk Assessment (QMRA), as described later in this article.

Water Sports

Water related sports may represent excellent relaxation activities, but can also be dangerous. They are often associated with superficial injuries, and in more serious cases, deep trauma, or even drowning; which can represent risk factors for several fungal infections. Fungi posing risks to people involved in water sports are species of *Cylindrocarpon*, *Microsporum*, *Phialemonium*, *Rhinocladiella*, and zygomycetous genera *Rhizopus*, *Mucor*, *Lichtheimia*, *Cunninghamella*, *Rhizomucor*, and *Saksenaea* (Novak Babič *et al.*, 2018). These fungi have been associated with a variety of infections from localized to disseminated, depending on the host's immune system. Fungi clearly connected to water-transmitted disease are those of *Scedosporium apiospermum* complex causing near-drowning syndrome, with characteristic cerebral abscesses or pulmonary infection developed after weeks of exposure.

In some areas of the globe, endemic fungi may be a problem for water sports. Sipsas and Kontoyiannis (2008) ran a meta-study to review cases of invasive fungal infections (IFI), looking into the lifestyle and contexts of the patients and concluded in terms of water sports in endemic areas for *Blastomyces* spp., that there is a certain amount of risk associated with the activity.

Cave Exploration

In 2018, a group of 12 members of a junior football team and their assistant-coach went cave exploring in Thailand and heavy rains caused a landslide trapping them in the cave for 17 days. Once rescued, they were all quarantined in an isolation room because of any potential infections myriad pathogens that can be transmitted in caves: Marburg hemorrhagic fever, leptospirosis (Weil's disease), rabies, borreliosis (cave fever), melioidosis (Whitmore's disease), and histoplasmosis (Gorvett, 2018).

Bats inhabit caves and their excreta, named guano, is a breeding ground for many micro- and macroorganisms. Bat guano inside caves in endemic areas for *Histoplasma capsulatum* pose an infection threat by this fungus. The fungus causes histoplasmosis, also known as "cave disease", and is well known in North and Central America as a cause of pneumonia. This histoplasmosis is often misdiagnosed, which leads to an inappropriate therapeutic approach with antibiotics. Immunocompetent individuals have

about a 5% chance of developing the disease when exposed to environmental levels of the fungus. These odds change to between 50% and 100% chance of getting the infection when exposed to higher levels of the fungus, like in caves.

Histoplamosis has a second, disseminated, stage of the disease, which can happen many years after the primary infection. *H. capsulatum* survives intracellularly in the reticuloendothelial system and may be reactivated mainly (not exclusively) with old age or immune-suppression (Ashbee *et al.*, 2008). Management of the disseminated histoplasmosis, if successful, is achieved by administration of systemic anti-fungal therapy.

In the United States of America, *H. capsulatum* inhabits the soil in the eastern and central parts of the country, especially in the Ohio and Mississippi River Valleys. It is however not exclusive to North and Central America. It has been found in South America, Africa, India, and Southeast Asia (Ashbee *et al.*, 2008).

Gardening and Camping

Gardening is a wonderful opportunity to commune with nature but some of the plants that we deal with have structures that may pierce our skin. *Sporothrix*, the infectious agent causing sporotrichosis, or rose gardener's disease as it is commonly known, is an IFI. *Sporothrix* is one of the most famous agents associated with gardening but also in terms of occupational exposure; it seems to affect people working with tree nursing, garden centers, forestry and hay bale handling (CDC, 2019). Sporotrichosis has a higher prevalence in tropical and temperate climates despite being present in all the world. Japan, India, Mexico, Brazil, Uruguay, Peru, and United States (associated with pine seedlings and moss manipulation (Barros *et al.*, 2011)) are endemic areas for this fungus. *Scopulariopsis brevicaulis*, *Saksenaea vasiformis*, *Scedosporium prolificans*, and *Exophiala jeanselmei* have also been found as minority gardening IFI agents (Sipsas and Kontoyiannis, 2008).

Different species of plants and trees seem to be connected also with symptoms known under the term "farmer's lung disease". The causative agents of the disease are highly irritant spores and mycotoxins wallimidione, walleminone and walleminol (walleminol A) produced by *Wallemia* spp. These fungi are xerophilic, osmophilic, halophilic, and chaophilic and can thrive on pollen, dry plant material, like hay or leaves, or in the presence of highly concentrated salts or sugars. Spores disperse in large quantities via air during hot and dry weather periods (Zajc and Gunde-Cimerman, 2018).

Lastly, *Cryptococcus gattii sensu lato* is making its way around the world, including Europe, despite being previously considered a tropical and subtropical climate fungus (Hagen *et al.*, 2012). The number of cases is increasing; there are several genotypes known and from those it has been concluded that some of those cases are allochthonous but a high percentage (40%) are autochthonous, many of which are in the Mediterranean region. This yeast can be found in decaying vegetable matter primarily, but it has also been isolated from animals and soil. It can stay dormant in its hosts and manifest itself much later. Unlike its close relative, the opportunistic *Cryptococcus neoformans*, *Cryptococcus gattii sensu lato* is a pathogen. *C. gattii* causes a pneumonia-like infection, with persistent cough, shortness of breath, chest pain and fever and if it spreads to the brain, patients get a cryptococcal meningoencephalitis, experiencing headache, fever, neck pain, nausea, and vomiting, sensitivity to light, confusion or changes in behavior (CDC, 2015). Susceptibility tests *in vitro* suggest that different genotypes react differently to various antifungals. Therapy may involve a combination of Amphotericine B with Fluconazol (Chen *et al.*, 2014) and management may require corticosteroids and draining in order to relieve the intracranial pressure (Franco-Paredes *et al.*, 2015). A more recent approach recommends a triplet of antifungals, lipid Amphotericin B formulation and 5-Flucytosine for 2–6 weeks followed consolidation/maintenance therapy with fluconazole for 12 months or longer.

As mentioned above, *Fusarium* spp. is a common fungus in the ground and care should be taken with rubbing eyes in order to prevent fungal keratitis, by this but also by other fungi. Soil is full of microbes, many of which are fungal opportunists and allergenic taxa, as mentioned above relative to swimming pools (*Alternaria*, *Aspergillus*, *Bipolaris*, *Cladosporium*, *Curvularia*, *Helminthosporium*, *Epicoccum*, *Aureobasidium*, *Penicillium*, *Malassezia*, *Saccharomyces cerevisiae*, and *Trichophyton*). Susceptible campers must take care and avoid camping sites with moist ground in order to avoid turning a camping event into a trip to hell.

Sandboxes

Undoubtedly, where there is sand and there are children, there will be a confluence of both. Children love to build structures on sand, like castles and dams (depending on water availability); they like to dig and even to bury themselves and others, often ending up eating some of the sand. 190 mg/day was the estimate of sand ingested by children without pica (extraordinary tendency to eat sand – van Wijnen *et al.*, 1990) at the beach. There is usually concomitant animal life on sand, especially outside of beach visitation hours. Considering that children get sand on their skin, eyes, ears, hair, nails and mucosae, and that prowling animals, rodents and birds shed whatever they have on their skin besides leaving excreta behind, it is easy to envision why sand should be monitored for pathogens.

Municipalities and public health protection offices have occasionally expressed concerns about microbial contaminants in sandboxes of public parks and beaches. A good example is a publication by European researchers with the result of such worries, in and around Lisbon, on sandboxes from three elementary schools and two kindergartens. The results were unsettling, but not necessarily surprising: *"Potentially pathogenic fungi were isolated from all samples besides one. Fusarium dimerum (32.4%) was found to be the dominant species in one park and Chrysonilia spp. in the other (46.6%). Fourteen different species and genera were detected, and no*

dermatophytes were found. Of a total of 14 species and genera, the fungi most isolated from the samples of the elementary schools were Penicillium spp. (74%), Cladophialophora spp. (38%) and Cladosporium spp. (90%). Five dominant species and genera were isolated from the kindergartens. Penicillium spp. was the only genus isolated in one, though with remarkably high counts (32,500 colony-forming units per gram). In the other kindergarten Penicillium spp. *were also the most abundant species, occupying 69% of all the fungi found."* (*Fusarium dimerum* has changed to *Bisifusarium dimerum* (Lombard *et al.*, 2015; Viegas *et al.*, 2016). The authors therefore recommend that sand be cleaned and replaced regularly.

Beach sand was addressed in another heading in this article (See Section "Sunbathing"). The different matrices pose different concerns, since the microorganisms at the beach tend to be less concentrated than in sandboxes.

Climate Change – The Game Changer

The days of geographically circumscribed endemic fungal infections are over. Until not many years ago, if you were a physician practicing somewhere in Europe and you got a patient with pneumonia, you would probably not suspect an endemic fungal infection like blastomycosis or histoplasmosis, unless your patient traveled recently to North America and mentioned it. Generally, endemic fungi remain endemic for now, but maintain the potential for changes in range associated with climatic alterations. The problem is that known pathogens and opportunists may experience new niches, displacement or range expansion due to extreme weather events associated with climate change and in concert with globalization (McIntyre *et al.*, 2017). This may include endemic fungi, as already may have happened in the Pacific Northwest region of North America, with a Vancouver Island outbreak caused by *Cryptococcus deuterogattii* which has historically been endemic to tropical and subtropical climates (Kidd *et al.*, 2004). There is still some controversy over the origin of this pathogen, which some authors believe to have originated in a mutant lineage originating somewhere else in the West-coast of North America (Ma *et al.*, 2009). More recently, however, a new taxonomy for this *C. gatii* complex was proposed by Hagen *et al.* (2015).

There is a known movement of air carrying fungal particles from Africa to America (Kellogg and Griffin, 2003), no doubt associated with the jet streams that flow westbound and converge at the equator. This may have been the cause of propagation of the plant pathogen *Hemileia vastatrix* (Bowden *et al.*, 1971) responsible for coffee leaf rust, a fungal infection devastating entire crops of coffee in South America since 1970. McIntyre *et al.* (2017) may have focused on the general alterations of climate because of the expansion of high temperatures beyond their usual latitudes. However, together with globalization, extreme weather events are generating the biggest concerns and the strangest clinical cases. The following three examples illustrate the worries about climate change and extreme weather events:

(1) Central Europe has reported bacterial *Vibrio cholerae* necrotizing fasciitis in bathers of inland water catchments (Hirk *et al.*, 2016);
(2) There are cases of mucormycosis associated with wood fragments flying at high speeds during hurricane-like winds (Neblett Fanfair *et al.*, 2012);
(3) A case of mucormycosis associated with catastrophic floods with a near-drowning situation, in Mandra, Attica in Greece, in November 2017 (Sympardi *et al.*, 2019).

Cases like these suggest that public health protection agencies need to advise the public on susceptibility factors more actively during extreme weather events and for climate change preparedness. For that, knowledge gathering is crucial. Benedict and Park (2014) prepared a review on published cases of natural disasters and fungal infections. In this review, coccidioidomycoses were the leading infections with over 100 cases, both in a 1977 dust storm and the 1994 Northridge earthquake in the USA. Many of these kinds of situations, however, take place not only due to natural disasters but frequently in recreational settings (Sipsas and Kontoyiannis, 2008).

Quantitative Microbial Risk Assessment (QMRA)

In a public health context, and especially in the face of climate change and globalization that can lead to changes in fungal contaminant ranges, it will be important to look toward understanding human health outcomes resulting from exposure to fungi. Quantitative Microbial Risk Assessment (QMRA) can be a powerful tool for understanding how contaminant concentrations are related to risks to human health in areas like gardens, beaches, swimming pools, and caves.

QMRA relies upon contaminant concentration data, combined with an expected exposure pathway from the environment to the human host (**Fig. 1**), to determine the concentration (exposure dose) of contaminants that humans are likely to be exposed to during their activities. From there, QMRA applies that calculated exposure dose to an existing dose-response model. This model then predicts the probability of a health response (e.g., infection, illness, death, etc.), given the calculated exposure dose (**Fig. 2**, Haas *et al.*, 2014). This probability of a health response is also known as the "risk" in quantitative microbial risk assessment and can be used to inform management and policy guidance for controlling contamination in the local environment.

Recent research has indicated that bacterial and viral health risks at beaches can be substantial, but that they are also highly dependent upon both the concentration of the bacteria or viruses and the exposure pathway. For instance, it has been found that

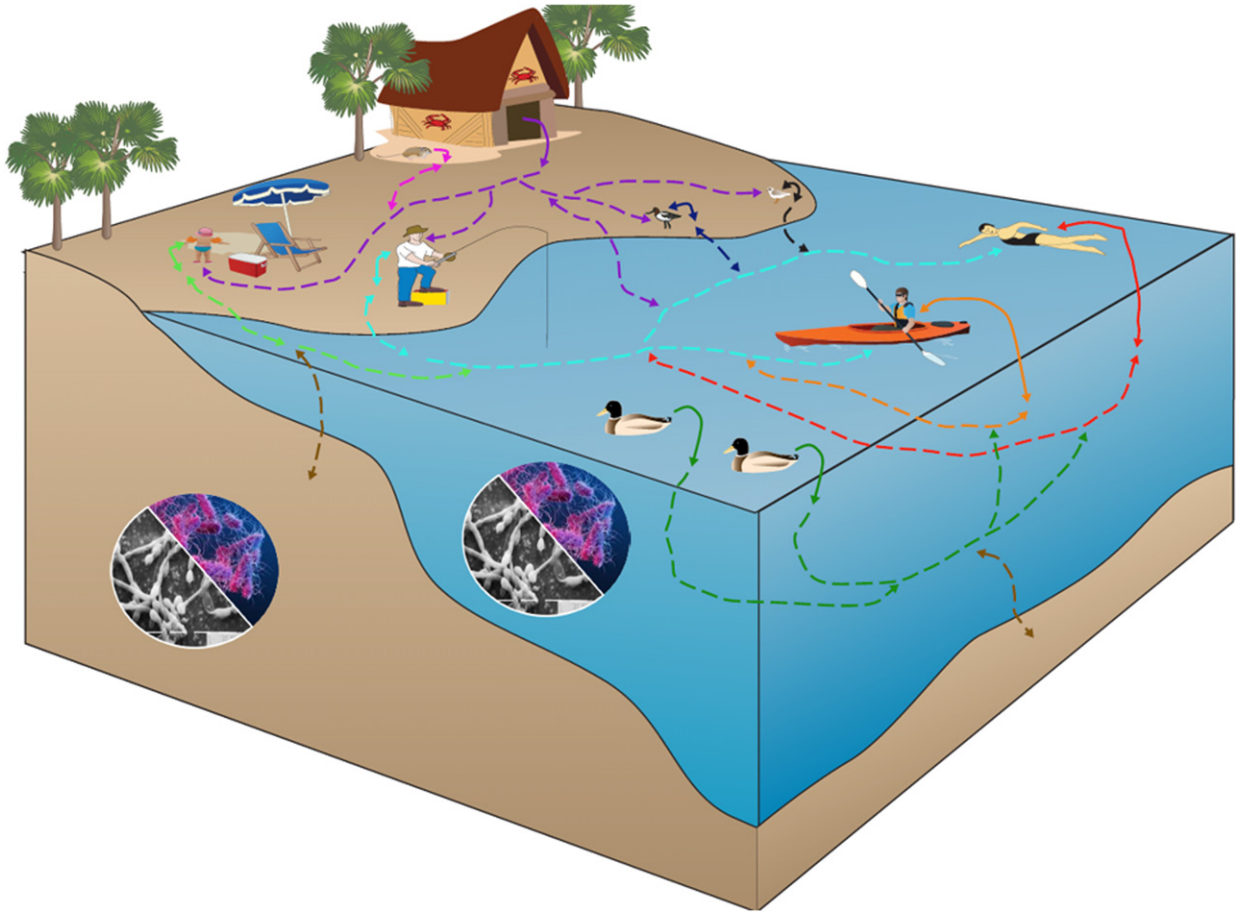

Fig. 1 Potential exposure pathways that human and animal hosts may be subject to at the beach. Different colors represent distinct pathways. Dashed lines represent transport of contaminants through the water or sand media; solid lines denote transfer of contaminants between the environment and human or animal hosts. Image created using vector graphics from Tracey Saxby, Kim Kraeer, Lucy Essen-Fishman, Jane Thomas, and Kate Moore, Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/imagelibrary/) and images of microorganisms from the US Centers for Disease Control and Prevention. Reproduced from Jabra-Rizk, M.A., Falkler, W.A., Meiller, T.F., 2004. Fungal biofilms and drug resistance. Emerging and Infectious Diseases 10 (1), 14–19. doi:10.3201/eid1001.030119.

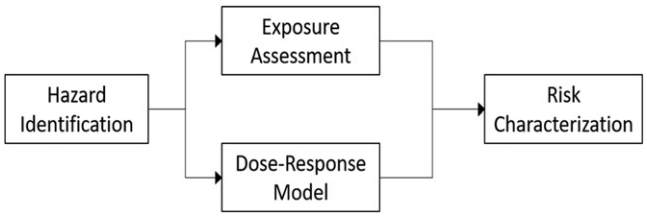

Fig. 2 General flow of development for a QMRA. Contaminant concentration observations are used in hazard identification and exposure assessment, while exposure pathways from the environment to human hosts are used in the exposure assessment. Epidemiological or feeding study observations are used with exposure assessment results in the dose-response modeling process, to calculate overall risk.

contaminant concentrations can be much higher in sand than in recreational water (Heaney *et al.*, 2012), due to the accumulation of contaminants in biofilms (Piggot *et al.*, 2012). However, the overall health risks associated with sand contamination may be comparable to or less than those associated with water contact because of the more complex exposure pathways that sand contaminants must take to infect humans.

A variety of QMRAs have been developed for recreational water in beach settings (Dickinson *et al.*, 2013; Eregno *et al.*, 2016; Juntunen *et al.*, 2017; Shibata and Solo-Gabriele, 2012; and others) and swimming pools (Peters *et al.*, 2017). These risk assessments, however, are exclusively in the context of contamination by bacteria or viruses; there is very little risk assessment work that is focused on fungal contamination in recreational areas. QMRAs developed for mycotoxin in agricultural environments

(Wu, 2004; Han *et al.*, 2013) may provide examples to draw from in developing fungal QMRAs for recreational settings. However, their applicability is limited because of the sensitivity of human health outcomes and risks to differences in both the microbial taxon and the setting/exposure pathway. It will therefore be crucial for ongoing research to focus on characterizing exposure pathways and developing epidemiologically-based dose-response models for fungal contaminants in areas like beaches, swimming pools, spas, gardens, and caves.

Conclusion

Currently, there is a rising awareness of the global burden of fungal infections. Bongomin *et al.* (2017) estimated nearly 2 billion people are suffering from a hair, skin or nail infection and tens of millions of from mucosal candidosis. They also describe the main fungal agents responsible for these infections at a global scale: *"Although the epidemiology of fungal diseases has greatly changed over the past few decades, Aspergillus, Candida, Cryptococcus species, Pneumocystis jirovecii, endemic dimorphic fungi such as Histoplasma capsulatum and Mucormycetes remain the main fungal pathogens responsible for the majority cases of serious fungal disease. Candida albicans is the main agent responsible for mucosal disease, Aspergillus fumigatus for most allergic fungal disease and Trichophyton* spp., *especially T . rubrum, for skin infections."* According to the same study, 150 million people suffer from serious fungal infection. Many of which are in less healthy economies but serious fungal infections have one common denominator throughout the whole world: the survival rate of the patient starts, *grosso modo*, at 50% and ends in 0% (Hagen, 2018). Mortality is currently over 1.6 million, which is similar to tuberculosis and three times that of malaria. Despite these numbers, there is no policy to fight fungal infections by health authorities anywhere but the WHO recently included two pathologies in the tropical neglected diseases list: Chromoblastomycosis and Mycetoma (WHO – World Health Organization, 2016).

One of the weak points in public policy management of fungal infections is neglecting their treatment rate and not considering fungi as a group of diverse pathologies and infection agents. This posture would make sense considering that all fungal infections are handled with a handful of antimicrobial agents and approaches. The biochemistry of our cells, of protozoan parasites and of fungi is very similar due to small evolutionary distances, so there are not many options for selectively treating an infection, leaving the patient unharmed.

The best policy in public health protection from fungal infections is by preventing them instead of managing or treating them, given the low treatment efficacies. However, it has been called a neglected epidemic (Armstrong-James *et al.*, 2014) in the particular context of HIV/AIDS patients but likely for others as well.

This article focused on exposure in leisure contexts, and the main goal of the authors is to raise awareness for the possible fungal ailments associated with leisure activities in the most common practices and contexts, because awareness leads to prevention and management.

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Chapter 5

Fungal contaminants in water and sand: A new frontier for quantitative microbial risk assessment

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Fungal contaminants in water and sand: A new frontier for quantitative microbial risk assessment

Chelsea J. Weiskerger[1] and João Brandão[2,3]

Abstract

Water sports and recreation can lead to exposure to microbial pathogens, including opportunists. At the beach, the Guidelines for Safe Recreational Water Environments by the World Health Organization are currently the leading public health and safety regulatory recommendations. As policy-makers begin to revisit the Guidelines for Safe Recreational Water Environments and subsequent legislation, consideration of evidence gathered by a global community of varied professional fields is recommended.

Quantitative microbial risk assessment is a recent approach that estimates the risk of exposure to pathogens in recreational water from dose–response relationships and observed pathogen concentrations. This can be powerful in informing public health policy in recreational areas. However, some microorganisms, namely fungi, do not yet have established median infectious doses, despite their known ability to impact human health at beaches. Can we calculate the risk of fungal exposure? Not yet, but we should work toward this goal.

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Introduction

Recreational aspects of water in both natural and man-made environments, such as spas and swimming pools, have been well documented since early civilizations [1]. However, as the scientific community has expanded its knowledge regarding microbial contamination in recreational settings, ensuring water quality and safety of recreational users has required increasingly complex and multivariate approaches.

Beaches are popular destinations for local residents and tourists across the globe. In the face of heavy beach usage, it is critical for beach managers to ensure that both water and sand are safe for contact with humans [2]. For decades, beach managers and researchers have tracked the presence and concentrations of pathogenic and indicator microbial taxa in recreational waters [3]. In many cases, *Escherichia coli* or enterococci are used as indicators of contamination at beaches, but they may not tell the entire story of beach safety and beachgoers' health [4–6].

There has been substantial discussion about the suitability of the current indicator taxa as proxies for pathogenic contamination at beaches. In addition, microbes causing disease beyond gastroenteritis, including those causing skin and respiratory ailments, have recently gained attention for their impact on human health [4,7]. Opportunistic microbes, including many fungi, have been observed on recreational beaches [8], potentially contributing to additional health risks to beachgoers in coastal areas. Study of contamination at recreational areas has largely remained concentrated on bacterial and viral taxa of human health concern, notwithstanding the prevalence of these other microbes. Similarly, contamination of sand at beaches has received little regulatory focus, relative to contamination in recreational water. This is in spite of research suggesting that sand may be a substantial source of beach contamination [3,4,9–12].

A signal of a potential shift in focus may be the decision by the World Health Organization to update Chapter 6, on sand contaminants, during the 2020 review of its Guidelines for Safe Recreational Water Environments. These guidelines are the reference document for bathing water regulation around the world and Chapter 6 was last edited in 2003 [13]. A revision is therefore currently underway to update the chapter in light of research and knowledge gained since 2003. The authors of this viewpoint wish to contribute to the general knowledge pool with a specific subject to be addressed by a

proximal approach. Herein we present the case for recognizing fungal and opportunistic contaminants and their roles in recreational water quality and beach health. We also emphasize the disconnection between existing quantitative microbial risk assessments (QMRAs) and the emerging opportunistic and pathogenic fungal contaminants. This leads to the suggestion that additional research should focus on obtaining the data necessary to include these emerging contaminants in future QMRAs. Previous work has alluded to the impact that fungi can have on human health at recreational areas [7,11,14], but this is the first work that connects these potential microorganisms of human health concern to QMRA as a way to assess risks associated with them.

Why fungi?

One of the understudied biological groups as sand contaminants are fungi. This group comprises heterotrophs known to act as biological recyclers in nature. Many fungal taxa are opportunists to human health, causing ailments when conditions are favorable. It is known that the immune system can limit the fungal invasion only to a certain extent but this competence tends to change during our lives, and with external factors and conditions. For example, yeast infections can capitalize on the use of certain medications (e.g. broad-spectrum or long-term antibiotic therapies and steroids) as well as debilitating viral infections (e.g. flu and HIV primo-infection) and other underlying disorders (e.g. diabetes mellitus). Yet, not all clinically-relevant fungal taxa are opportunists. Some are actual pathogens; dermatophytes, for example, cause ringworm, tineae and onychomycosis, and endemic/dimorphic fungi can cause severe and life threatening invasive fungal infections (regardless of the immune status of the host). *Coccidioides immitis* and *Coccidioides posadasii*, *Histoplasma capsulatum* and *Histoplasma duboisii*, *Blastomyces dermatitidis*, *Cryptococcus deuterogatii*, *Paracoccidioides brasiliensis*, *Talaromyces marneffei*, and *Sporothrix schenckii* species complex are the currently described endemic fungi, responsible respectively for coccidioidomycosis or valley fever, histoplasmosis (American and African), blastomycosis, cryptococcosis, Paracoccidioidomycosis, Talaromycosis, and Sporotrichosis. Expressing a low prevalence, even during outbreaks, endemic mycosis agents have no known median infectious doses. They all are associated with water-rich environments, in some way, except coccidioidomycosis and sporotrichosis, which are associated with desert sand, and plants and pets, respectively. Nonetheless, inhalation of spores and fungal particles is likely the most common environmental exposure route for fungi, regardless of setting, as shown in Table 1. Inland beaches in endemic areas are therefore areas of concern for many of these fungal infections.

Also of concern for those who are susceptible are fungal allergies. A study by Tanaka et al. on modelling of the immunological reaction to exposure to *Aspergilli* calculated a responsive increase in cytokines almost a full day after exposure, triggering a consequent inflammatory response [15]. Assuming the compatibility of the model with other allergenic-inhaled fungi, this delay in symptom development may lead to difficulty in associating an exposure with an outcome, rendering clarification of the role that beaches play as exposure sites difficult.

Alongside beaches, surfaces at swimming pools and water parks are known to be propagation environments for many fungi, some of which are pathogenic. Dermatophytes are just one example of pathogenic fungi, causing athlete's foot and other superficial infections. These recreational facilities can stimulate the growth of fungi that resist chlorination in water, and the warm temperatures favor dematiaceous fungi [16], some of which are toxin-producing and allergenic [17]. One of the worries about this kind of environment is the susceptibility of some recreational users, including the elderly, users with diabetes and other immunologic or metabolic deficiencies, and severe asthma. In addition, there are taxa that naturally occur in watery environments and that cause severe opportunistic infections. This is the case for *Fusarium* spp. that may cause fungal keratitis or fusariosis [18]. Natural Mucorales are also highly associated with wet ground and known to cause fatal infections during natural disasters and violent storms [19,20].

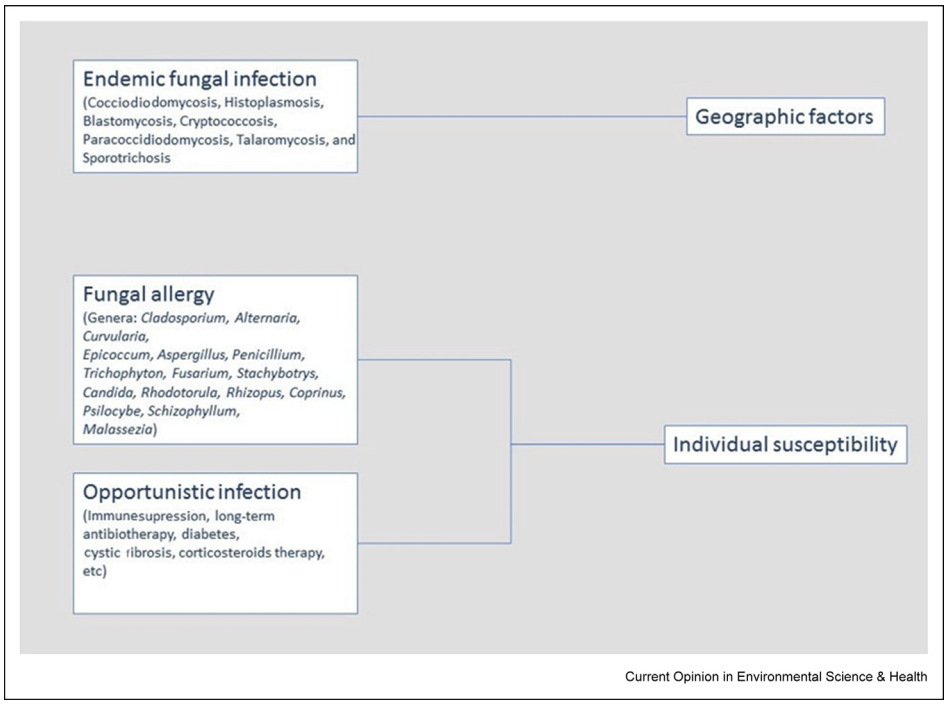
Figure 1 shows the possible concerns regarding exposure to fungi, influenced by two very different weighting factors: geography and host susceptibility. Table 1 shows a list of the major fungal infectious agents in humans, their respective pathologies and natural habitat.

A crash course in QMRA

Because of the public health implications of recreational water and sand quality, QMRA has become a valuable tool for researchers. QMRA can help connect the various contamination sources at beaches and other settings, to health concerns resulting from localized contamination.

QMRA frameworks for recreational areas use microbial contaminant concentrations collected *in situ*, knowledge of exposure pathways from observation or literature, and *in vivo* epidemiological information about exposure doses and their resulting health effects (dose–response [22]) as input data. From these data and knowledge bases, probability distributions can be developed for the calculation of exposure and risk terms such as ingestion volume during exposure ($v_{ingestion}$), exposure dose ($d_{exposure}$; Eq. (1); [22]), and probability (or risk) of infection ($P_{infection}$, Eq. (2); [22]).

Figure 1



Exposure to fungi in beach sand with respective major influencing aspects.

$$d_{exposure} = v_{ingestion} * C_{contaminant} \quad (1)$$

$$P_{infection} = 1 - e^{-k*d_{exposure}} \quad (2a)$$

$$P_{infection} = 1 - \left[1 + d_{exposure}\frac{\left(2^{1/\alpha} - 1\right)}{N_{50}}\right]^{-\alpha} \quad (2b)$$

Here, $C_{contaminant}$ denotes the contaminant concentration (generally in terms of MPN/100 mL of water, or MPN/g of sand, depending on medium), $d_{exposure}$ represents the exposure dose (MPN), and $v_{ingestion}$ represents a known average volume of ingestion from pilot studies (MPN/h, MPN/day, and MPN/exposure event) [23]. k denotes the probability of contaminant survival to the point of infection, N_{50} represents the dose at which 50% of a population is expected to be affected (median infectious dose, MPN/kg), and α is a parameter derived from the beta distribution used to model variable contaminant survival probability.

Although the initial collection of contaminant concentrations used in QMRA work is straightforward, the development of exposure pathways and an exposure assessment, is less direct. Exposure assessments involve evaluation of the exposure pathway from the contaminant source to the target as well as the frequency, duration, and magnitude of a typical exposure to the source of the contaminant by the target host. These components of an exposure assessment may vary in their complexity because of the differential complexity in the exposure routes themselves. In an example of QMRA, in the context of exposure to contaminants from beach sand, an exposure assessment may include factors like sand-to-hand and hand-to-mouth transfer efficiencies for the contaminant, skin surface area, sand-to-skin adherence efficiency, and hand-to-mouth touching frequency [24]. Alternatively, an exposure assessment in the context of exposure to contaminants via whole-body contact in recreational water would depend only on an assumed volume of water ingested per time and a known or assumed period of contact with the water [25–27]. Other exposure- and contaminant-dependent factors can also complicate the development of an effective QMRA, especially when knowledge of these factors from observations is limited. Such complicating factors can include whether the target host of the QMRA is an adult or a child, host body size and weight, strength of the host's immune system, contaminant decay rates in the environment, and whether the QMRA itself is based on a snapshot in time or time-dependent. It is crucial that research continue to characterize not only these

Table 1

List of the major fungal infectious agents in humans, their respective pathologies and natural habitat (Centers for Disease Control and Prevention, USA; Leading International Fungal Infection (LIFE), UK; Mycology Online, University of Adelaide, Australia).

Fungi	Disease/role	Pathogenicity	Sources	Exposure route [21]
Aspergillus spp.	Aspergilloma, aspergillosis, onychomycosis, allergy	Opportunist	Environment	Direct contact/inhalation
Blastomyces dermatitidis	Blastomycosis	Pathogen	Decaying vegetable matter	Inhalation
Candida spp.	Candidosis	Opportunist	Gut, skin, and mucosae of animals and environment	Direct contact
Cladophialophora bantiana	Cerebral phaeohyphomycosis	Pathogen	Soil and rotten plant material	Inhalation
Coccidioides spp.	Coccidioidomycosis	Pathogen	Soil	Inhalation
Cryptococcus spp.	Cryptococcal meningitis, pneumonia, and/or systemic infection	Opportunist/pathogen	Decaying vegetable matter and bird feces	Inhalation
Dermatophytes	Onychomycosis, tineae	Pathogen	Skin, hairs, nails, and environmental (some taxa)	Direct contact
Exophiala spp.	Phaeohyphomycosis	Opportunist/pathogen	Decaying wood and soil enriched with organic waste	Inhalation
Fonsecaea spp.	Chromoblastomycosis	Opportunist	Decaying wood and soil enriched with organic waste	Inhalation
Fusarium spp.	Fusariosis	Opportunist	Water and vegetable matter	Inhalation/aspiration/ direct contact
Geotrichum spp.	Geotrichosis	Opportunist	Environment and animal flora	Inhalation
Histoplasma capsulatum	Histoplasmosis	Pathogen	Guano of birds and bats	Inhalation
Madurella spp.	Mycetoma	Opportunist	Soil	Direct contact
Mucor spp.	Mucormycosis (zygomycosis)	Opportunist	Decaying wood and soil enriched with organic waste	Direct contact
Paracoccidioides brasiliensis	Paracoccidioidomycosis	Pathogen	Unknown	Inhalation
Pneumocystis jirovecii	Pneumocystosis	Opportunist	Water, air, carrier individuals	Inhalation
Rhizopus spp.	Mucormycosis (zygomycosis)	Opportunist	Decaying wood and soil enriched with organic waste	Inhalation
Rhodotorula spp.	Fungemia, endocarditis, meningitis, endophthalmitis, peritonitis	Opportunist	Environment and animal flora	Ingestion
Sporothrix schenckii	Sporotrichosis	Opportunist	Environment and pets	Inhalation
Talaromyces marneffei	*Talaromycosis*	Pathogen	Bamboo rats and humans	Inhalation
Trichosporon spp.	Trichosporonosis	Opportunist	Environmental	Direct contact

influences but also others that may not be recognized yet, through observational data, to optimize exposure assessments used in QMRAs.

Final calculation of probability of infection can involve the application of exponential (Eq. (2a)), beta-Poisson (Eq. (2b)), or other statistically-derived dose–response models to the exposure doses calculated in the exposure assessment process. Frequently, QMRA development relies on the use of existing dose–response relationships from *in vivo* epidemiological or feeding studies, rather than developing new

Table 2

Recently published (2015–2020) existing complete QMRA case studies in the context of beaches or swimming pools.

Geographical context	Target microbial taxon	Target host	Exposure activity	Target medium	Reference
Marine beaches in South Florida, USA	*Cryptosporidium* spp., *Staphylococcus aureus,* Enterovirus	Children (aged <18 years)	Playing in sand, directly ingesting sand	Beach sand	Shibata and Solo-Gabriele [24]
Marine beaches in Southern California, USA	*Enterococcus* spp., Fecal Coliform	Adults	Surfing	Recreational water	Tseng and Jiang [34]
Marine beaches in California, USA	*E. coli* O157:H7, *Campylobacter* spp., *Salmonella* spp., *Cryptosporidium* spp., *Giardia* spp., Norovirus	Adults and children	Swimming	Recreational water	Brown et al. [35]
Marine beaches in Southern California, USA	*Vibrio cholerae, Vibrio, parahaemolyticus, Vibrio vulnificus*	Adults and children	Swimming, surfing	Recreational water	Dickinson et al. [36]
Marine beaches in Northern California, USA	*Giardia* spp., Adenovirus	Adults and children	Swimming, surfing	Recreational water	Seto et al. [37]
Marine beaches in Denmark	Enterotoxigenic *E. coli, Campylobacter jejuni, Giardia lamblia*	Adults	Swimming	Recreational water	Andersen et al. [38]
Marine beach in Puerto Rico	*Enterococcus* spp., *Bacteroidales* spp., *Clostridium perfringens,* F+ coliphage, Norovirus, Adenovirus, *Cryptosporidium* spp., *Giardia* spp., *E. coli* O157:H7, non-O157:H7 Shiga toxin producing *E. coli*, *Campylobacter* spp., *Salmonella* spp.	Adults and children	Swimming	Recreational water	Soller et al. [25]
Marine beaches in Southern Norway	*Campylobacter* spp., *Salmonella* spp., *Cryptosporidium* spp., *Giardia* spp., Norovirus	Adults and children	Swimming	Recreational water	Eregno et al. [39]
River and marine beach sites in Southern California, USA	Adenovirus	Adults and children	Swimming, playing in sand	Recreational water and sand	Kundu et al. [40]
Marine beaches in Southern California, USA	Norovirus, Adenovirus, Enterovirus, *Campylobacter jejuni, Salmonella enterica*	Adults	Surfing	Recreational water	Soller et al. [41]
Marine beaches in Taiwan	Enterococci	Adults and children	Swimming	Recreational water	Jang and Liang [42]
Freshwater beaches in Philadelphia, PA, USA	Enterococci	Adults	Jet skiing, canoeing, kayaking, boating, fishing, wading, playing in water, swimming, tubing, water skiing	Recreational water	Sunger and Haas [43]
Freshwater beaches in Uganda	Norovirus, Rotavirus, *Campylobacter* spp., *Salmonella* spp., *E. coli*, *Cryptosporidium* spp., *Ascaris lumbricoides*	Adults and children	Swimming	Recreational water	Fuhrimann et al. [44]

(*continued on next page*)

Table 2 (*continued*)

Geographical context	Target microbial taxon	Target host	Exposure activity	Target medium	Reference
Freshwater beaches in China	*Salmonella enterica, Mycobacterium avium*	Adults and children	Rowing, fishing, walking	Recreational water	Fang et al. [45]
Marine beaches in Brazil	Diarrheagenic *E. coli*	Adults and children	Swimming	Recreational water	Rodrigues et al. [46]
Freshwater beaches in China	*Cryptosporidium* spp. and *Giardia* spp.	Adults and children	Swimming, rowing, wading	Recreational water	Xiao et al. [47]
Recreational river water in Virginia, USA	*Campylobacter jejuni, E. coli* O157:H7, *Cryptosporidium* spp., *Giardia lamblia, Salmonella enterica, N*orovirus	Adults	Swimming	Recreational water	Liao et al. [48]
Recreational river water in Ontario, Canada	*Cryptosporidium parvum, Cryptosporidium hominis*	Children	Swimming	Recreational water	Lapen et al. [49]
Recreational river water in China	*Cryptosporidium* spp. and *Giardia* spp.	Adults and children	Swimming	Recreational water	Xiao et al. [50]
Recreational freshwater in Singapore	Norovirus, Adenovirus	Adults and children	Dragon boating, kayaking, fishing, picnicking	Recreational water	Vergara et al. [51]
Recreational river waters in South Africa	Norovirus	Adults and children	Swimming, boating	Recreational water	Van Abel et al. [52]
Recreational river waters in Argentina	Rotavirus	Adults and children	Swimming	Recreational water	Prez et al. [53]
Recreational marine and freshwater areas in the Netherlands	*Vibrio* spp.	Adults and children	Swimming	Recreational water	Sterk et al. [54]
Freshwater beaches in China	*Salmonella* spp., *Mycobacterium avium, Pseudomonas aeruginosa*	Adults and children	Boating, playing in water, feeding fish, walking, visiting a decorative fountain	Recreational water	Cui et al. [45]
Swimming pools in the Netherlands	*E. coli* O157:H7, *Salmonella enterica, Campylobacter jejuni*	Adults and children	Swimming	Swimming pool water	Peters et al. [55]
Swimming pools in Arizona, USA	*Cryptosporidium* spp.	Adults and children	Swimming	Swimming pool water	Suppes et al. [56]

relationships. The resulting dose–response models are the core predictors of risk, and depend on factors such as the microbial taxon, target host that is exposed to the microorganism, the medium from which the target host is exposed, and the health response type [22]. The probability of infection calculated from the exposure dose and dose–response model indicates both how many people would be expected to contract an infection from a single exposure event and the overall probability that a single person gets infected from a single exposure. These probabilities can then be compared to risk thresholds to determine whether environments are safe for contact or if they should be closed to visitors.

Because of high levels of variability in the types of data on which dose–response models are based, it is important to conduct epidemiological studies on exposure and dose–response for individual microorganism taxa. However, high fiscal and temporal costs, as well as ethical considerations, can make such epidemiological and feeding studies difficult. As a result, there is a considerable lack of contemporaneous dose–response data overall, but particularly for emerging contaminants in recreational settings [28–31]. The limited availability of these types of data can dictate whether QMRAs can be performed for specific taxa, regardless of the need for or usefulness of such assessments. For instance, there has been very little epidemiological research to-date on dose–response relationships for fungal taxa of human health concern [32], as much of the research on fungal epidemiology remains focused on treatment. The development of QMRAs for fungal contaminants has lagged behind the growth of bacteria- and virus-based

QMRAs because of this lack of dose–response data, to the potential detriment of public health in recreational areas.

Connecting emerging concerns to QMRA approaches for beach management

To date, QMRAs and their epidemiological dose–response data have focused on bacteria, viruses, protozoa, and prions (Table 2; [22,33]). As research into recreational water quality and contamination has progressed in recent years, though, the prevalence of emerging contaminants like opportunistic microorganisms, including fungi, has become clear. Consequently, there is a substantial gap in data and knowledge of the human health effects of opportunistic and fungal contamination at beaches, especially in relation to those health effects of historically-studied pathogenic taxa. Without those data and knowledge, QMRAs in the context of fungal contamination in recreational environments will be severely limited in their predictions and applicability.

Beyond recreational beach settings, fungal contamination has been established as a substantial source of infection from the use of swimming pools and water parks [57–59]. Because of their inherent disinfection processes, it is often assumed that swimming pools and water parks are not sources of contamination or infection. However, some fungal contaminants have shown a potential resistance to disinfection techniques used in these settings [58,60]. This can lead to their persistence and a propensity to infect swimmers under otherwise disinfected conditions. Not only has existing QMRA work largely excluded fungal contaminants as a whole, but Table 2 indicates that it has also often omitted swimming pool and water park settings, with Suppes et al. [56] and Schets et al. [61] serving as exceptions.

Similarly, QMRAs associated with recreational areas have largely been developed for exposures associated with recreational water contact. Recently, however, it has become clear that the entire beachscape—including water, sand, submerged sediments, and hard surfaces at swimming pools or water parks—can harbor contaminants of human health concern [11]. Therefore, it is important to not only consider emerging contaminants but also to assess health risks linked with exposure to contamination from sources beyond the water.

QMRA development can benefit from additional research; perhaps most importantly, the expansion of exposure and epidemiological study of fungal impacts on human health. Opportunistic and pathogenic fungi are a frequently-overlooked form of contamination, especially in recreational areas that are more focused on bacterial and viral pollutants. As a result, there are very limited existing QMRAs in the context of fungal contamination in recreational areas. However, their impact on human health and their potential prevalence as recreational contaminants warrant additional research that will foster their inclusion in future QMRAs for beaches, swimming pools, and other recreational areas.

Conclusions

There is a need for QMRA frameworks focusing on both emerging contaminants and exposure pathways associated with media beyond recreational water at beaches, to more fully understand the human health risks connected to beach usage and other recreational water settings. This provides a great opportunity for advancements in the study of fungal and opportunistic pathogen exposure pathways and exposure doses leading to health effects. It also opens up the prospect of developing QMRAs for such emerging contaminants that accumulate throughout the beachscape, including recreational water, sand, and submerged sediment. Currently, the field of QMRA is expanding from recreational water, to include risk assessments for exposures from sand at the beach. However, there are no existing QMRAs that can speak to the risks associated with exposure to fungal contaminants in recreational settings, to the authors' knowledge. By expanding the applicability of QMRA to opportunistic microorganisms, like most fungi, research can better inform the system-scale management of recreational areas. Such an expansion will account for risks associated with exposure to a wider variety of microorganisms of human health concern. This can, in turn, help to characterize and minimize the diffuse risks of health effects from a day at the beach, spa, pool, or water park and maximize the safety and enjoyment of recreational area usage in all of its forms.

Conflict of interest statement

Nothing declared.

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Chapter 6

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

Brandão, J., Gangneux, J.P., Arikan-Akdagli, S., Barac, A., Bostanaru, A.C., Brito, S., Bull, M., Çerikçioğlu, N., Chapman, B., Efstratiou, M. A., Ergin, Ç., Frenkel, M., Gitto, A., Gonçalves, C.I., Guégan, H., Gunde-Cimerman, N., Güran, M., Irinyi, L., Jonikaitė, E., Kataržytė, M., Klingspor, L., Mares, M., Meijer, W.G., Melchers, W.J.G., Meletiadis, J., Meyer, W., Nastasa, V., Novak Babič, M., Ogunc, D., Ozhak, B., Prigitano, A., Ranque, S., Rusu, R.O., Sabino, R., Sampaio, A., Silva, S., Stephens, J.H., Tehupeiory-Kooreman, M., Tortorano, A.M., Velegraki, A., Veríssimo, C., Wunderlich, G.C., Segal, E.

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Mycosands: Fungal diversity and abundance in beach sand and recreational waters — Relevance to human health

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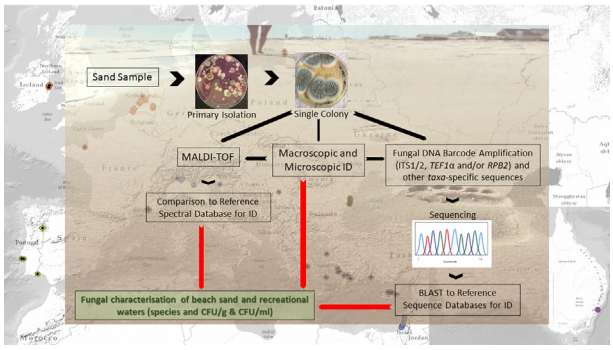
HIGHLIGHTS

- 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.

GRAPHICAL ABSTRACT



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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-retractions. 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, allergenic fungi, 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.

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 *Microsporum*, 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 sensitization (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% (Latgé 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; Latgé 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 (Levetin et al., 2016).

Endemic fungi of potential interest to sand exposure are *Histoplasma*, *Coccidioides*, *Paracoccidioides*, *Blastomyces*, *Sporothrix*, *Emergomyces* and *Talaromyces marneffei*. 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; Ulfig 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 **Regions**, as shown in Fig. 1 (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;(GR): 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, Akbük, Ören, Akyaka, İztuzu, Sarıgerme, 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 Fig. 1 with QGIS (Ver 3.10.0-A Coruña) which is a free licensed GIS application (GNU General Public License CC BY-SA).

2.2. Analytical procedures

2.2.1. Sample preparation and incubation

Sand: Dry (supratidal) sand samples (between 100 and 200 g, between 5 and until 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 min at 100 rpm and the extract was then plated (0.2 ml) in triplicates per media (Sabouraud's Dextrose agar (SDA) and Mycosel agar (Cyclohexamide, 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 underwater (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 1α (*TEF1α*) or the RNA polymerase II gene (*RPB2*)) fungal DNA barcodes and other taxa specific primers, further detailed per participating laboratory in the supplementary material (table S8), 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

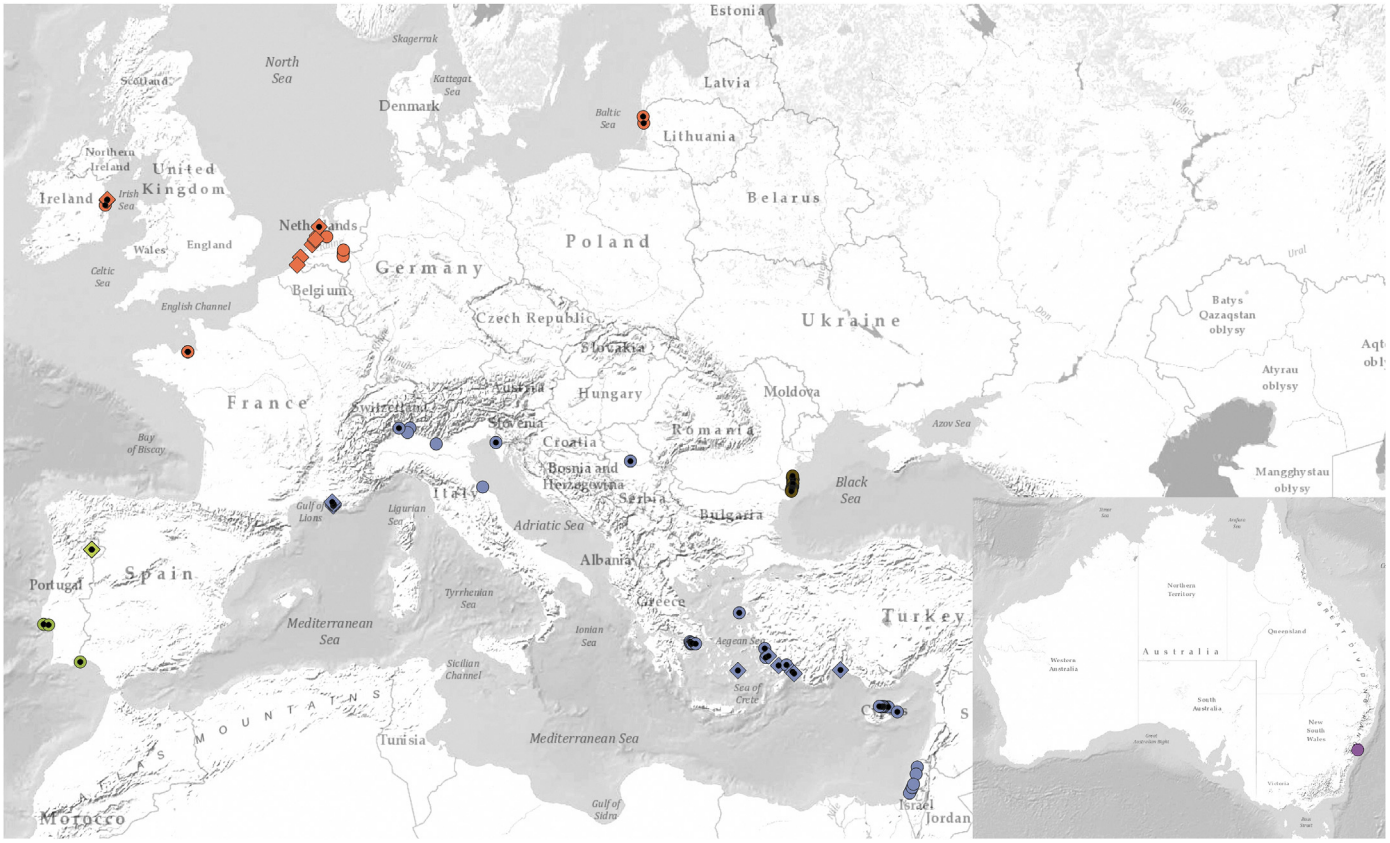

Fig. 1. Geographical distribution of the sampling points using mapping with QGIS (Version 3.10.0-A Coruña). Circles correspond to urban beaches and diamonds to non-urban beaches. Dots within the shapes indicate water-sampling sites. Red = Northwest Europe, Green = Southwest Europe, Blue = Mediterranean, Brown = Black Sea and Purple = Sydney (Australia). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 (table S9).

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 (freshwater shores and coastal beaches); type of sand (mix and pure sand); for regions of countries aggregated by geographical and climatological proximity (Black Sea, Mediterranean, Northwest 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 *Microsporum, Trichophyton*, *Arthroderma*, *Epidermophyton* and (unidentified) Dermatophytes; **'Allergenic fungi'** – contains all species of fungi excluding Yeasts and Dermatophytes; **'Dematiaceous fungi'**: contains all fungi with melanin-like pigments in the cell walls as described above, described by 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

Fig. 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.

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.

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 *Nigri* (<0.001), *Candida* spp. ($p = 0.027$), *Rhodotorula* spp. ($p < 0.001$), *Cryptococcus* spp. (0.0191), '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 least (myco-) populated ones at the beach.

During this project, the median relative humidity is highest in the Northwest Europe region (78.0 (60.0, 97.0) %) and lowest in Sydney

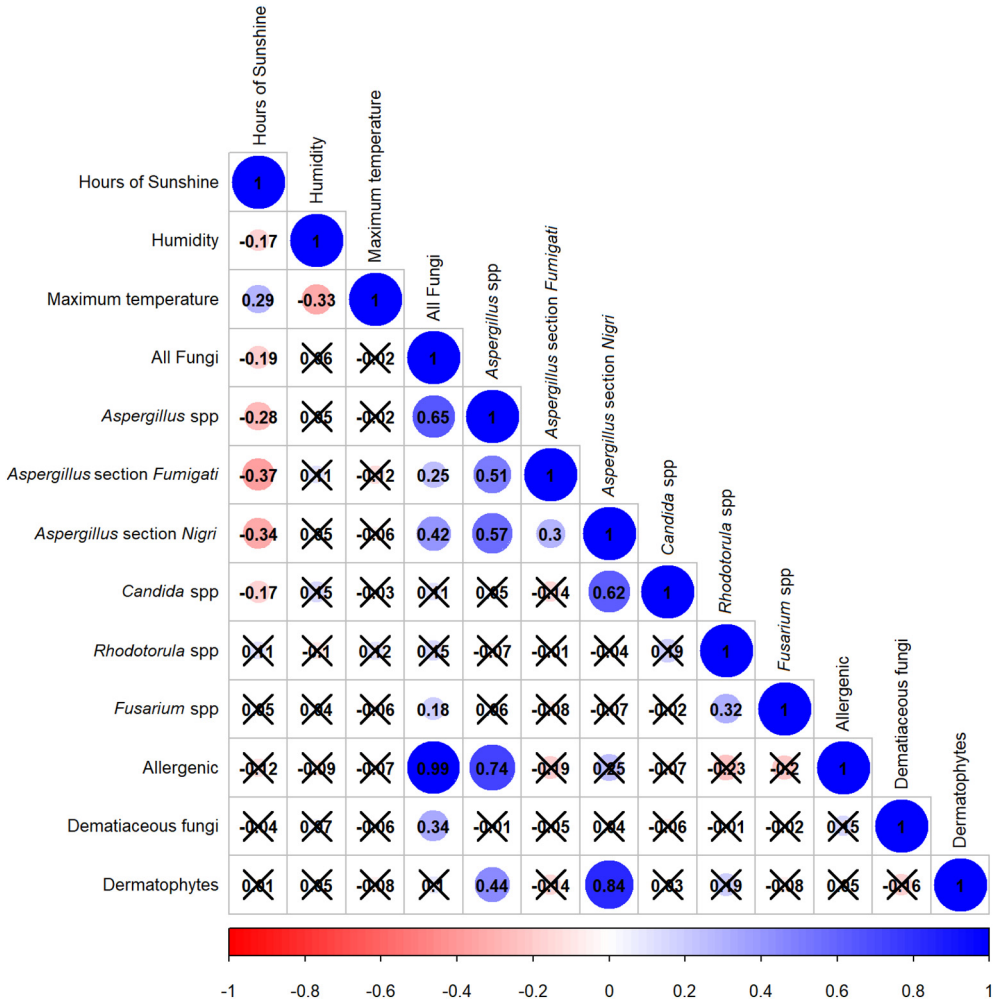

Fig. 2. Sand correlations between fungal parameters, maximum temperatures during sampling and hours of sunshine (statistically significant correlations do not have an X on top of the value).

(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 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**
Aspergillus 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
Aspergillus section *Fumigati*	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
Aspergillus section *Nigri*	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
Candida 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
Candida albicans	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
Candida parapsilosis	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
Candida tropicalis	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
Candida dubliniensis	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**
Rhodotorula 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
Cryptococcus 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
Fusarium 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

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 (83.31533.3)	**<0.001**
Aspergillus 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**
Aspergillus section *Fumigati*	NA	0.0 (0.0, 425.0)	0.0 (0.0, 91.7)	0.0 (0.0, 16.7)	NA	
Aspergillus section *Nigri*	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**
Aspergillus section *Flavi*	NA	0.0 (0.0, 2000.0)	NA	NA	NA	
Candida 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**
Candida albicans	0.0 (0.0, 20.0)	0.0 (0.0, 50.0)	NA	NA	NA	
Candida parapsilosis	0.0 (0.0, 30.0)	0.0 (0.0, 30.0)	NA	NA	0.0 (0.0, 123.3)	
Candida tropicalis	NA	0.0 (0.0, 10.0)	0.0 (0.0, 41.7)	NA	NA	
Candida dubliniensis	NA	NA	1.7 (0.0, 516.7)	NA	NA	
Rhodotorula 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**
Cryptococcus 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**
Fusarium 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	

Note: N = number of samples.

(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 = 160.5 CFU/g and 213.7 CFU/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.

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
Aspergillus 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
Aspergillus section *Fumigati*	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
Aspergillus section Nigri	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
Candida 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
Candida albicans	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
Candida parapsilosis	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
Candida tropicalis	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
Candida dubliniensis	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
Rhodotorula 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
Cryptococcus spp.	NA	NA		NA	NA		NA	NA	
Fusarium 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

3.2. Water

3.2.1. Pearson's correlation

Fig. 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.

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).

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, 83.3) 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 $p < 0.0011$.

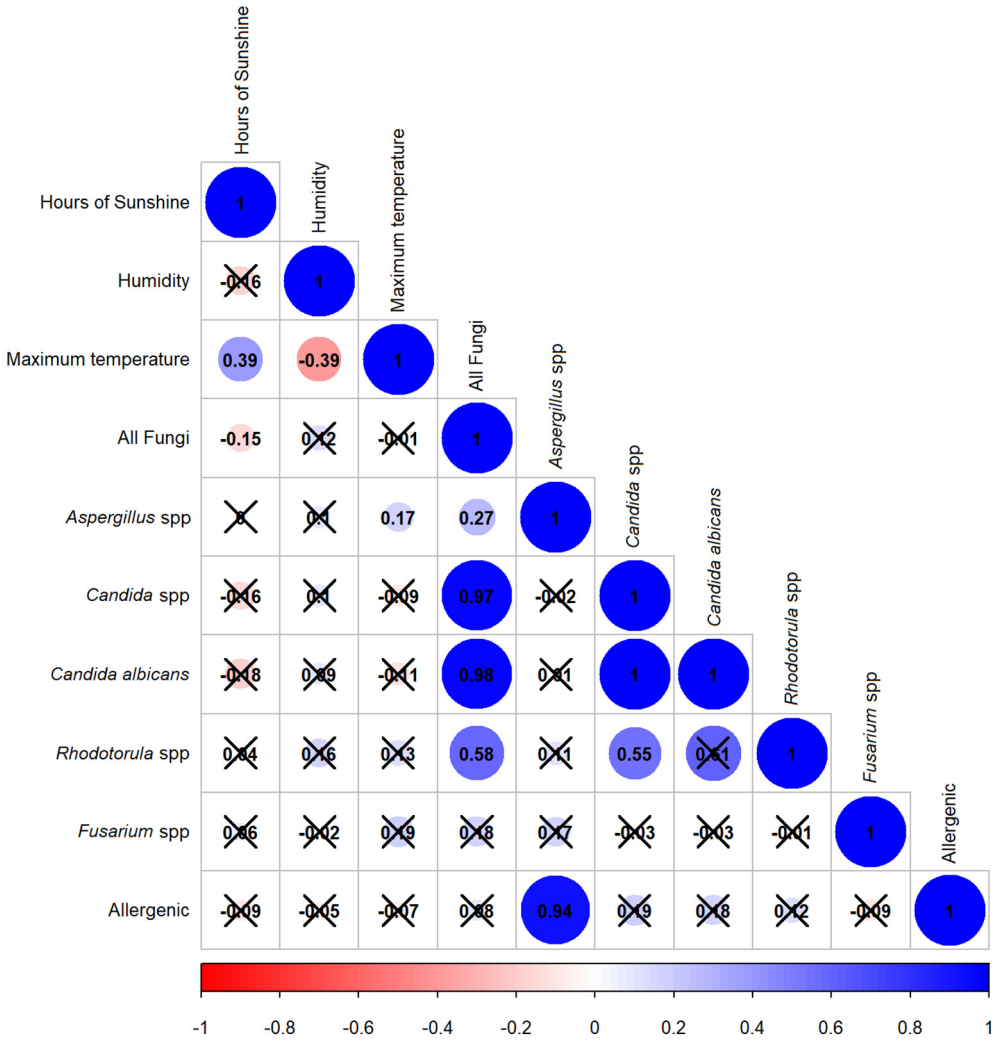

Fig. 3. Water correlations between fungal parameters, maximum temperatures during sampling and hours of sunshine (statistically significant correlations do not have an X on top of the value).

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
Aspergillus spp.	NA	0.0 (0.0, 201.7)	0.0 (0.0, 181.7)	0.0 (0.0, 83.3)	**0.0041**
Aspergillus section *Fumigati*	NA	0.0 (0.0, 20.0)	0.0 (0.0, 90.0)	NA	
Aspergillus section *Nigri*	NA	0.0 (0.0, 41.7)	NA	0.0 (0.0, 83.3)	
Aspergillus section *Flavi*	NA	0.0 (0.0, 0.7)	NA	NA	
Candida spp.	0.0 (0.0, 2.0)	0.0 (0.0, 12.0)	0.0 (0.0, 1585.0)	NA	
Candida albicans	NA	3.3 (0.0, 8.3)	0.0 (0.0, 170.0)	NA	
Candida parapsilosis	NA	6.0 (0.0, 12.0)	NA	NA	
Candida glabrata	NA	0.0 (0.0, 2.5)	0.0 (0.0, 1.7)	NA	
Candida tropicalis	0.0 (0.0, 1.0)	NA	0.0 (0.0, 90.0)	NA	
Candida dubliniensis	NA	NA	0.0 (0.0, 1325.0)	NA	
Rhodotorula 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
Fusarium 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, 83.3) 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 Mycosands 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. (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 and Baker (1984) reported from 1 to 15,900 CFU/g fungi from beach sand of Hawaii. Much smaller numbers were observed by Echevarría (2019) from the sand of beaches of Costa Rica (6 to17 CFU/g), but they were isolating only filamentous fungi. Salvo and Fabiano (2007) isolated 0 to 11.470 CFU/g of yeasts and 58–1.778 CFU/g of filamentous fungi in the northwestern Italian beaches. 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 Błaszak, 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., 2005; Gomes et al., 2008; Kishimoto and Baker, 1969; Londono et al., 2018; Muntañolacvetković and Ristanović, 1980; Müller, 1973; Papadakis et al., 1997; Rudenko et al., 2011; Salvo and Fabiano, 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. (2012) who, investigating the correlation of human beach use to the abundance of fungi in the sand, describe *Rhodotorula mucilaginosa* and *R. sloofiae* only in marine beaches heavily used by humans, while 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 (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 dermatophytosis 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. de Moura Sarquis and de Oliveira (1996) and 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. (2012) and 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 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 30–35 °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 (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 *Microsporum 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 temperatures 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 and Baker (1984), Gomes et al. (2008), and 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, Cladosporium, Coniochaeta, Curvularia, Didymella, Epicoccum, Exophiala, Exserohilum, Fonsecaea, Hortaea, 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 Góralska, 2016; Brandão et al., 2010; Falcão et al., 1993; Góralska et al., 2020; Kiziewicz et al., 2004; Sherry et al., 1979; Wójcik et al., 2003).

While 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 then from coastal waters ($p < 0.001$). This could be attributed (as in the case of sand) to environmental conditions being more favourable to their 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. (1987) isolated 30–1020 CFU/50 ml yeasts from marine beach water, Aulicino et al. (2001) from 1 to 10^3/100 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. (2003) who isolated from 8 to 32,800 CFU/100 ml yeasts from reservoir water used for recreational bathing. Góralska et al. (2020) detected an average of 40 to 460 CFU/100 ml 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. (1997) claim that yeasts of human origin correlated with the numbers of swimmers in seawater. Velegraki-Abel et al. (1987), also investigating in Greek coastal bathing waters, reported that yeast counts increased during the summer. 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 Góralska 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 (25× 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 Góralska, 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. (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 were 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. (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 colonizers. 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.

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Additional information

The original draft introduction was written in sections: 'Framing fungi in sand' by JB and WGM; The yeasts genera *Candida*, *Cryptococcus*, *Trichosporon*, *Geotrichum* by ES; 'Allergenic fungi' moulds and endemic fungi by JPG and SR; Black mould by MNB and NG-C; and Dermatophytes by RS and CV. Statistical analysis was performed by SS, supported by JB, ES, JPG and MNB and reviewed by ÇE. The original draft "Conclusions" were written by ES and JB and the original draft "discussion", by MAE and JB. ÇE generated the map of all sampling *loci*.

CRediT authorship contribution statement

Conceptualization: JB, JPG and ES; **Methodology:** JB, JPG, MB, NÇ, BC, AG, WGM, WM, SR, RS, SS, JHS, AMT, CV, GCW and ES; **Software:** SS; **Validation:** JB, MB, BC, AG, LI, WJGM, WM, AP, SS, JHS, AMT, AV, GCW and ES; **Local data analysis:** JB, JPG, AB, ACB, SB, MB, NÇ, BC, MAE, ÇE, MF, AG, CIG, HG, NG-C, MG, LI, MK, LK, MM, WGM, WJGM, JM, WM, VN, MNB, DG, BO, SR, ROR, RS, AS, JHS, MTK, AV, CV, GCW and ES; **Final formal analysis:** JB, JPG, ÇE, MNB, SS and ES; **Investigation:** JB, JPG, AB, ACB, SB, NÇ, MAE, ÇE, MF, AG, CIG, HG, NG-C, MG, EJ, MK, LK, MM, WGM, WJGM, JM, VN, MNB, DO, BO, AP, SR, ROR, RS, AS, JHS, MTK, AV, CV, GCW and ES; **Resources:** JB, ACB, SB, MB, NÇ, BC, ÇE, MF, CIG, HG, MG, LI, MK, LK, WGM, JM, WM, VN, MNB, AP, SR; ROR, AS, AMT, AV, GCW and ES; **Data Curation:** JB, MF, AG, LI, WGM, WJGM, MNB, AP, SR, RS, SS, JHS and GCW; **Writing - Original Draft:** JB, JPG, MAE, ÇE, NG-C, WGM, MNB, SR, RS, SS, CV and ES; **Writing - Review & Editing:** JB, JPG, SA-A, AB, ACB, SB, MB, NÇ, BC, MAE, ÇE, MF, AG, CIG, HG, NG-C, MG, LI, EJ, MK, LK, MM, WGM, WJGM, JM, WM, VN, MNB, DO, BO, AP, SR, ROR, RS, AS, SS, JHS, MTK, AMT, AV, CV, GCW and ES; **Visualization:** JB, JPG, ÇE, WM, MNB, SS and ES; **Supervision:** JB, JPG MB, BC, LI, WM and ES; **Central project administration:** JB, JPG and ES; **Local Project administration:** JB, JPG, SA-A, BC, NG-C, MK, MM, WGM, WJGM, WM, SR, AS, AMT, AV and ES; **Funding acquisition:** JB, JPG, BC, MM, WM, AS, AV and ES.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.146598.

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Supplemental Text for

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

Table S1: Number of samples by matrix (sand or water) per country:

Country	AU	IE	FR	GR	IL	IT	LT	NL	PT	RO	SL	RS	TR	Total
Sand	9	31	19	51	17	13	10	13	34	80	4	9	82	372
Water	0	31	19	45	0	1	10	1	34	80	4	9	82	316

Note: 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

Table S2: Number of sampling events per country for sand samples per region

Sand - number of samples														
Country	AU	IE	FR	GR	IL	IT	LT	NL	PT	RO	SL	RS	TR	Total
Black Sea	0	0	0	0	0	0	0	0	0	80	0	0	0	80
Mediterranean	0	0	15	19	17	13	0	0	0	0	9	4	82	159
Northwest Europe	0	51	16	0	0	0	10	13	0	0	0	0	0	90
Southwest Europe	0	0	0	0	0	0	0	0	34	0	0	0	0	34
Sydney (Australia)	9	0	0	0	0	0	0	0	0	0	0	0	0	9

Note: 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

Table S3: Number of sampling events per country for water samples per region

Water - number of samples												
Country	IE	FR	GR	IT	LT	NL	PT	RO	SL	RS	TR	Total
Black Sea	0	0	0	0	0	0	0	80	0	0	0	80
Mediterranean	0	15	19	1	0	0	0	0	9	4	82	130
Northwest Europe	45	16	0	0	10	1	0	0	0	0	0	72
Southwest Europe	0	0	0	0	0	0	34	0	0	0	0	34

Note: 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

Table S4: Relative humidity per region

Median (range) in %					
Variable	**Mediterranean (N=159)**	**Northwest Europe (N=90)**	**Southwest Europe (N=34)**	**Sydney, Australia (N=9)**	**P value**
Relative humidity	64.0 (19.0, 98.0)	78.0 (60.0, 97.0)	70.0 (25.0, 96.0)	60.0 (60.0, 67.0)	**< 0.001**

Table S5: Number of samples with fungi in sand samples by country (CFU/g)

Sand														
	AU (N=9)	FR (N=31)	GR (N=19)	IE (N=51)	IL (N=17)	IT (N=13)	LT (N=10)	NL (N=13)	PT (N=34)	RO (N=80)	RS (N=4)	SL (N=9)	TR (N=82)	Total (N=372)
Fungi (all)														
N	9	31	19	51	17	13	10	13	34	80	4	9	82	372
Mean (SD)	624.1 (527.3)	46.5 (168.3)	1051.1 (1010.6)	61.1 (99.8)	158.6 (214.8)	78.2 (104.6)	1127.4 (1122.4)	144.2 (338.5)	542.6 (1181.9)	208.5 (340.7)	310.0 (301.7)	1203.9 (450.0)	308.8 (366.9)	321.3 (610.3)
Median(Range)	366.7 (83.3,1533.3)	0.0 (0.0,928.3)	650.0 (57.5,3365.0)	20.0 (0.0,581.7)	40.0 (5.0,647.0)	16.7 (0.0,300.0)	1002.5 (3.3,3497.5)	15.0 (0.0,1207.5)	90.8 (1.7,6400.0)	72.5 (0.5,2170.0)	262.5 (16.7,698.3)	1166.7 (630.0,2268.3)	166.7 (0.0,1583.3)	89.2 (0.0,6400.0)
***Aspergillus* spp**														
N	6	31	19	51	15	13	10	2	34	0	4	9	70	264
Mean (SD)	296.7 (425.3)	15.9 (38.0)	716.2 (867.6)	5.5 (9.3)	73.7 (139.0)	21.8 (38.1)	3.2 (3.2)	40.0 (49.5)	107.5 (261.5)	NA	123.3 (103.2)	367.0 (188.5)	84.3 (153.3)	117.5 (325.6)
Median(Range)	48.3 (0.0,943.3)	0.0 (0.0,156.7)	310.0 (50.0,2930.0)	1.7 (0.0,40.0)	5.0 (0.0,430.0)	0.0 (0.0,116.7)	2.5 (0.0,10.0)	40.0 (5.0,75.0)	1.7 (0.0,900.0)	NA	124.2 (16.7,228.3)	323.3 (116.7,748.3)	33.3 (0.0,833.3)	5.0 (0.0,2930.0)
Aspergillus* section *Fumigati														
N	0	31	13	0	9	13	10	2	8	0	0	0	20	106
Mean (SD)	NA	10.0 (26.4)	60.2 (137.3)	NA	7.2 (10.9)	10.3 (22.1)	3.1 (3.1)	13.8 (15.9)	2.1 (5.9)	NA	NA	NA	13.3 (37.3)	15.4 (54.5)
Median(Range)	NA	0.0 (0.0,91.7)	0.0 (0.0,425.0)	NA	0.0 (0.0,25.0)	0.0 (0.0,66.7)	2.5 (0.0,10.0)	13.8 (2.5,25.0)	0.0 (0.0,16.7)	NA	NA	NA	0.0 (0.0,166.7)	0.0 (0.0,425.0)
Aspergillus* section *Nigri														
N	3	0	19	0	6	13	0	2	34	0	0	9	64	150
Mean (SD)	568.9 (471.1)	NA	174.2 (245.3)	NA	46.7 (107.0)	11.5 (21.9)	NA	26.2 (33.6)	46.4 (164.5)	NA	NA	2.8 (8.3)	54.6 (155.4)	70.6 (184.7)
Median(Range)	723.3 (40.0,943.3)	NA	95.0 (5.0,950.0)	NA	2.5 (0.0,265.0)	0.0 (0.0,66.7)	NA	26.2 (2.5,50.0)	0.0 (0.0,833.3)	NA	NA	0.0 (0.0,25.0)	0.0 (0.0,833.3)	0.0 (0.0,950.0)
***Candida* spp**														
N	6	0	0	51	12	0	10	1	10	24	4	9	28	155
Mean (SD)	31.1 (51.8)	NA	NA	29.2 (85.1)	19.3 (38.3)	NA	1.0 (2.4)	5.0 (NA)	2.2 (6.9)	4.6 (11.0)	27.1 (26.7)	6.7 (11.2)	19.0 (54.0)	17.8 (56.7)
Median(Range)	0.0 (0.0,123.3)	NA	NA	1.7 (0.0,555.0)	5.0 (0.0,105.0)	NA	0.0 (0.0,7.5)	5.0 (5.0,5.0)	0.0 (0.0,21.7)	0.0 (0.0,40.0)	29.2 (0.0,50.0)	0.0 (0.0,30.0)	0.0 (0.0,250.0)	0.0 (0.0,555.0)

Candida albicans														
N	0	0	0	0	6	0	0	0	0	4	4	0	5	19
Mean (SD)	NA	NA	NA	NA	1.7 (2.6)	NA	NA	NA	NA	5.0 (10.0)	27.1 (26.7)	NA	3.3 (7.5)	8.2 (15.9)
Median(Range)	NA	NA	NA	NA	0.0 (0.0,5.0)	NA	NA	NA	NA	0.0 (0.0,20.0)	29.2 (0.0,50.0)	NA	0.0 (0.0,16.7)	0.0 (0.0,50.0)
Candida parapsilosis														
N	3	0	0	0	6	0	0	0	0	4	0	9	0	22
Mean (SD)	41.1 (71.2)	NA	NA	NA	4.2 (3.8)	NA	NA	NA	NA	7.5 (15.0)	NA	5.6 (10.1)	NA	10.4(26.7)
Median(Range)	0.0 (0.0,123.3)	NA	NA	NA	5.0 (0.0,10.0)	NA	NA	NA	NA	0.0 (0.0,30.0)	NA	0.0 (0.0,30.0)	NA	0.0 (0.0,123.3)
Candida glabrata														
N	0	0	0	51	0	0	0	0	0	0	0	0	0	51
Mean (SD)	NA	NA	NA	0.6 (1.8)	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.6 (1.8)
Median(Range)	NA	NA	NA	0.0 (0.0,8.3)	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.0 (0.0,8.3)
Candida tropicalis														
N	0	0	0	51	0	0	0	0	0	0	0	9	0	60
Mean (SD)	NA	NA	NA	3.1 (8.1)	NA	NA	NA	NA	NA	NA	NA	1.1 (3.3)	NA	2.8 (7.6)
Median(Range)	NA	NA	NA	0.0 (0.0,41.7)	NA	NA	NA	NA	NA	NA	NA	0.0 (0.0,10.0)	NA	0.0 (0.0,41.7)
Candida dubliniensis														
N	0	0	0	34	0	0	5	0	0	0	0	0	0	39
Mean (SD)	NA	NA	NA	37.2 (93.4)	NA	NA	0.5 (1.1)	NA	NA	NA	NA	NA	NA	32.5 (87.9)
Median(Range)	NA	NA	NA	3.3 (0.0,516.7)	NA	NA	0.0 (0.0,2.5)	NA	NA	NA	NA	NA	NA	1.7(0.0,516.7)
***Rhodotorula* spp**														
N	9	8	12	0	9	0	10	0	28	8	4	9	50	147
Mean (SD)	57.4 (59.6)	1.2(1.9)	43.3(143.8)	NA	2.2(2.6)	NA	19.5(43.1)	NA	0.2 (0.7)	13.2(37.5)	15.8 (29.5)	16.5(49.4)	183.0 (320.7)	73.0 (207.7)
Median(Range)	40.0 (0.0,166.7)	0.0 (0.0,5.0)	0.0 (0.0,500.0)	NA	0.0 (0.0,5.0)	NA	0.0 (0.0,130.0)	NA	0.0 (0.0,3.3)	0.0 (0.0,106.0)	1.7 (0.0,60.0)	0.0 (0.0,148.3)	0.0 (0.0,1333.3)	0.0 (0.0,1333.3)

***Cryptococcus* spp**														
N	6	4	0	0	5	0	10	0	10	0	4	0	0	39
Mean (SD)	40.6 (53.2)	125.0 (250.0)	NA	NA	14.0 (25.8)	NA	3.2 (7.0)	NA	0.2 (0.5)	NA	38.3 (41.3)	NA	NA	25.7 (83.3)
Median(Range)	13.3 (0.0,110.0)	0.0 (0.0,500.0)	NA	NA	5.0 (0.0,60.0)	NA	0.0 (0.0,22.5)	NA	0.0 (0.0,1.7)	NA	35.0 (0.0,83.3)	NA	NA	0.0 (0.0,500.0)
***Fusarium* spp**														
N	9	0	13	0	3	0	10	3	34	0	4	9	35	120
Mean (SD)	51.1 (40.6)	NA	22.3 (47.0)	NA	16.7 (28.9)	NA	2.5 (4.2)	36.7 (36.4)	4.2 (12.6)	NA	19.6 (25.7)	295.7 (107.3)	39.0 (86.3)	43.2 (94.0)
Median(Range)	33.3 (6.7, 123.3)	NA	0.0 (0.0, 150.0)	NA	0.0 (0.0, 50.0)	NA	0.0 (0.0, 10.0)	25.0 (7.5, 77.5)	0.0 (0.0, 68.3)	NA	10.8 (0.0, 56.7)	228.3 (180.0, 428.3)	16.7 (0.0, 400.0)	0.8 (0.0, 428.3)
Yeast														
N	9	8	15	51	17	0	10	2	28	52	4	9	56	261
Mean (SD)	105.6 (62.8)	63.7 (177.6)	44.7 (131.7)	29.2 (85.1)	18.9 (35.9)	NA	150.2 (393.9)	3.8 (1.8)	1.0 (4.2)	7.3 (19.5)	106.7 (112.5)	23.1 (48.2)	178.6 (316.5)	63.2 (187.0)
Median(Range)	123.3 (16.7,190.0)	0.0 (0.0,503.3)	0.0 (0.0,500.0)	1.7 (0.0,555.0)	5.0 (0.0,110.0)	NA	11.2 (0.0,1265.0)	3.8 (2.5,5.0)	0.0 (0.0,21.7)	0.0 (0.0,106.0)	90.0 (0.0,246.7)	0.0 (0.0,148.3)	0.0 (0.0,1333.3)	0.0 (0.0,1333.3)
Allergenic														
N	0	0	0	51	0	0	0	0	18	4	0	0	6	79
Mean (SD)	NA	NA	NA	27.5 (35.6)	NA	NA	NA	NA	970.7 (1507.2)	244.8 (160.2)	NA	NA	0.0 (0.0)	251.3 (808.7)
Median(Range)	NA	NA	NA	15.0 (0.0,170.0)	NA	NA	NA	NA	252.5 (50.0,6400.0)	310.0 (9.0,350.0)	NA	NA	0.0 (0.0,0.0)	21.7 (0.0,6400.0)
Dematiaceous fungi														
N	9	31	19	0	17	13	10	13	34	0	0	9	82	237
Mean (SD)	10.7 (13.5)	0.1 (0.6)	34.7 (89.7)	NA	1.2 (3.8)	15.4 (50.7)	258.8 (803.3)	27.7 (75.8)	71.0 (142.0)	NA	NA	7.2 (12.4)	17.1 (63.8)	32.9 (180.6)
Median(Range)	3.3 (0.0,36.7)	0.0 (0.0,3.3)	0.0 (0.0,350.0)	NA	0.0 (0.0,15.0)	0.0 (0.0,183.3)	1.7 (0.0,2545.0)	0.0 (0.0,275.0)	15.0 (0.0,600.0)	NA	NA	0.0 (0.0,31.7)	0.0 (0.0,416.7)	0.0 (0.0,2545.0)
Dermatophytes														
N	0	0	0	51	0	0	0	1	24	8	0	0	6	90
Mean (SD)	NA	NA	NA	4.4 (8.7)	NA	NA	NA	7.5 (NA)	7.6 (34.0)	25.0 (53.5)	NA	NA	34.7 (32.7)	9.1 (26.6)
Median(Range)	NA	NA	NA	1.7 (0.0,53.3)	NA	NA	NA	7.5 (7.5,7.5)	0.0 (0.0,166.7)	0.0 (0.0,150.0)	NA	NA	20.8 (0.0,83.3)	0.0 (0.0,166.7)

Table S6: Number of samples with fungi in water samples by country (CFU/ml)

Water												
	FR (N=31)	GR (N=19)	IE (N=45)	IT (N=1)	LT (N=10)	NL (N=1)	PT (N=34)	RO (N=79)	RS (N=4)	SL (N=9)	TR (N=82)	Total (N=315)
Fungi (all)												
N	31	19	45	0	10	0	34	79	4	9	82	313
Mean (SD)	7.5 (32.4)	8.5 (18.1)	39.5 (236.8)	NA	13.9 (28.1)	NA	13.5 (26.0)	5.2 (9.9)	159.6 (154.3)	12.4 (37.2)	23.2 (43.6)	18.6 (96.4)
Median(Range)	0.0 (0.0,181.7)	0.0 (0.0,62.0)	1.7 (0.0,1591.7)	NA	2.5 (0.0,91.7)	NA	4.2 (0.0,131.7)	0.0 (0.0,55.0)	161.7 (5.0,310.0)	0.0 (0.0,111.7)	0.0 (0.0,250.0)	0.0 (0.0,1591.7)
***Aspergillus* spp**												
N	31	19	45	0	10	0	34	0	4	0	70	213
Mean (SD)	7.4 (32.5)	4.8 (15.5)	0.8 (2.3)	NA	0.3 (1.1)	NA	3.4 (14.9)	NA	98.3 (94.2)	NA	10.0 (25.7)	7.4 (26.8)
Median(Range)	0.0 (0.0,181.7)	0.0 (0.0,62.0)	0.0 (0.0,13.3)	NA	0.0 (0.0,3.3)	NA	0.0 (0.0,83.3)	NA	93.3 (5.0,201.7)	NA	0.0 (0.0,133.3)	0.0 (0.0,201.7)
Aspergillus* section *Fumigati												
N	31	13	0	0	10	0	0	0	0	0	20	74
Mean (SD)	4.1 (16.1)	1.5 (5.5)	NA	NA	0.2 (0.5)	NA	NA	NA	NA	NA	0.8 (3.7)	2.3 (10.9)
Median(Range)	0.0 (0.0,90.0)	0.0 (0.0,20.0)	NA	NA	0.0 (0.0,1.7)	NA	NA	NA	NA	NA	0.0 (0.0,16.7)	0.0 (0.0,90.0)
Aspergillus* section *Nigri												
N	3	19	0	0	0	0	34	0	0	0	64	120
Mean (SD)	1.1 (1.9)	2.6 (8.1)	NA	NA	NA	NA	2.5 (14.3)	NA	NA	NA	2.5 (7.8)	2.5 (9.9)
Median(Range)	0.0 (0.0,3.3)	0.0 (0.0,30.0)	NA	NA	NA	NA	0.0 (0.0,83.3)	NA	NA	NA	0.0 (0.0,41.7)	0.0 (0.0,83.3)
***Candida* spp**												
N	0	14	45	0	10	0	0	24	4	0	0	97
Mean (SD)	NA	1.4 (3.6)	37.5 (236.0)	NA	0.2 (0.8)	NA	NA	0.1 (0.4)	3.8 (4.4)	NA	NA	17.8 (160.9)
Median(Range)	NA	0.0	0.0	NA	0.0	NA	NA	0.0	3.3	NA	NA	0.0

Candida albicans												
N	0	0	45	0	0	0	0	0	4	0	0	49
Mean (SD)	NA	NA	3.8 (25.3)	NA	NA	NA	NA	NA	3.8 (4.4)	NA	NA	3.8 (24.3)
Median(Range)	NA	NA	0.0 (0.0,170.0)	NA	NA	NA	NA	NA	3.3 (0.0,8.3)	NA	NA	0.0 (0.0,170.0)
Candida parapsilosis												
N	0	2	0	0	0	0	0	0	0	0	0	2
Mean (SD)	NA	6.0 (8.5)	NA	NA	NA	NA	NA	NA	NA	NA	NA	6.0 (8.5)
Median(Range)	NA	6.0 (0.0,12.0)	NA	NA	NA	NA	NA	NA	NA	NA	NA	6.0 (0.0,12.0)
Candida glabrata												
N	0	12	45	0	0	0	0	0	0	0	0	57
Mean (SD)	NA	0.2 (0.7)	0.1 (0.3)	NA	NA	NA	NA	NA	NA	NA	NA	0.1 (0.4)
Median(Range)	NA	0.0 (0.0,2.5)	0.0 (0.0,1.7)	NA	NA	NA	NA	NA	NA	NA	NA	0.0 (0.0,2.5)
Candida tropicalis												
N	0	0	45	0	0	0	0	4	0	0	0	49
Mean (SD)	NA	NA	2.6 (13.6)	NA	NA	NA	NA	0.2 (0.5)	NA	NA	NA	2.4 (13.0)
Median(Range)	NA	NA	0.0 (0.0,90.0)	NA	NA	NA	NA	0.0 (0.0,1.0)	NA	NA	NA	0.0 (0.0,90.0)
Candida dubliniensis												
N	0	0	30	0	5	0	0	0	0	0	0	35
Mean (SD)	NA	NA	46.0 (241.6)	NA	0.5 (1.1)	NA	NA	NA	NA	NA	NA	39.5 (223.7)
Median(Range)	NA	NA	0.0 (0.0,1325.0)	NA	0.0 (0.0,2.5)	NA	NA	NA	NA	NA	NA	0.0 (0.0,1325.0)
***Rhodotorula* spp**												
N	0	12	0	0	10	0	28	7	4	0	50	111
Mean (SD)	NA	0.8 (1.9)	NA	NA	0.8 (2.4)	NA	1.1 (4.8)	0.7 (1.9)	12.1 (19.0)	NA	9.3 (40.0)	5.1 (27.4)
Median(Range)	NA	0.0 (0.0,5.0)	NA	NA	0.0 (0.0,7.5)	NA	0.0 (0.0,25.0)	0.0 (0.0,5.0)	4.2 (0.0,40.0)	NA	0.0 (0.0,250.0)	0.0 (0.0,250.0)

***Cryptococcus* spp**												
N	0	0	0	0	0	0	0	0	0	0	0	0
Mean (SD)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Median(Range)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
***Fusarium* spp**												
N	0	0	0	0	0	0	34	0	4	0	35	73
Mean (SD)	NA	NA	NA	NA	NA	NA	0.0 (0.3)	NA	4.6 (5.3)	NA	1.4 (6.2)	1.0 (4.6)
Median(Range)	NA	NA	NA	NA	NA	NA	0.0 (0.0, 1.7)	NA	4.2 (0.0, 10.0)	NA	0.0 (0.0, 33.3)	0.0 (0.0, 33.3)
Yeast												
N	0	15	45	0	10	0	28	51	4	0	56	209
Mean (SD)	NA	2.0 (3.7)	37.5 (236.0)	NA	1.0 (2.4)	NA	1.1 (4.8)	0.5 (2.1)	28.3 (46.8)	NA	8.3 (37.9)	11.3 (111.4)
Median(Range)	NA	0.0 (0.0,12.0)	0.0 (0.0,1585.0)	NA	0.0 (0.0,7.5)	NA	0.0 (0.0,25.0)	0.0 (0.0,10.0)	7.5 (0.0,98.3)	NA	0.0 (0.0,250.0)	0.0 (0.0,1585.0)
Allergenic												
N	0	0	45	0	0	0	18	4	0	0	0	67
Mean (SD)	NA	NA	2.0 (2.6)	NA	NA	NA	16.2 (30.7)	0.5 (1.0)	NA	NA	NA	5.7 (17.0)
Median(Range)	NA	NA	1.7 (0.0,13.3)	NA	NA	NA	5.8 (0.0,131.7)	0.0 (0.0,2.0)	NA	NA	NA	1.7 (0.0,131.7)
Dematiaceous fungi												
N	31	19	0	0	10	0	34	0	0	9	82	185
Mean (SD)	0.1 (0.3)	0.1 (0.6)	NA	NA	0.5 (1.1)	NA	2.5 (5.0)	NA	NA	12.4 (37.2)	1.5 (8.4)	1.8 (10.1)
Median(Range)	0.0 (0.0,1.7)	0.0 (0.0,2.5)	NA	NA	0.0 (0.0,2.5)	NA		NA	NA	0.0 (0.0,111.7)	0.0 (0.0,66.7)	0.0 (0.0,111.7)
Dermatophytes												
N	0	0	45	0	0	0	0	0	0	0	0	45
Mean (SD)	NA	NA	0.0 (0.3)	NA	NA	NA	NA	NA	NA	NA	NA	0.0 (0.3)
Median(Range)	NA	NA	0.0 (0.0,1.7)	NA	NA	NA	NA	NA	NA	NA	NA	0.0 (0.0,1.7

Table S7: Opportunistic dematiaceous fungi isolated from sand and water during the study

Human disease	Fungal genera detected in beach sand with known pathogenic potential
Subcutaneous inf.	*Alternaria, Bipolaris, Cladophialophora, Cladosporium, Curvularia, Exophiala, Exserohilum, Fonsecaea, Microsphaeropsis, Phaeoacremonium, Phialophora, Phoma, Pyrenochaeta, Sporothrix*
Cutaneous inf.	*Alternaria, Chaetomium, Cladosporium, Cladophialophora, Exophiala, Exserohilum, Hortaea, Phialophora, Phoma, Scopulariopsis*
Endophthalmitis	*Microsphaeropsis, Pyrenochaeta*
Deep tissue inf.	*Cladophialophora, Exophiala, Fonsecaea, Microsphaeropsis, Pyrenochaeta*
Mycetoma	*Cladophialophora, Curvularia, Exophiala, Madurella, Neopyrenochaeta, Pyrenochaeta*
Allergic response	*Alternaria, Cladosporium*
Asthma	*Alternaria, Cladosporium*
Sinusitis	*Alternaria, Bipolaris, Coniochaeta, Curvularia, Epicoccum, Exserohilum*
Onychomycosis	*Alternaria, Curvularia, Scopulariopsis*
Keratitis	*Alternaria, Bipolaris, Curvularia, Exserohilum, Phialophora*
Peritonitis	*Aureobasidium, Bipolaris, Curvularia*
Neurotropic inf.	*Bipolaris, Chaetomium, Cladophialophora, Exophiala, Exserohilum, Fonsecaea*
Endocarditis	*Curvularia, Phialophora*
Systemic inf.	*Aureobasidium, Chaetomium, Curvularia, Exophiala, Phaeoacremonium, Pseudallescheria, Sporothrix*
Chromoblastomycosis	*Aureobasidium, Cladophialophora, Fonsecaea, Phialophora*
Phaeohyphomycosis	*Cladophialophora, Exophiala, Fonsecaea*
Pulmonary inf.	*Alternaria, Aureobasidium, Chaetomium, Cladophialophora, Curvularia, Exophiala, Pseudallescheria, Sporothrix*

Table S8: Pairs of primers and respective PCR conditions:

Region	Primer pair	Amplification (100ng DNA, 50ng of each primer, 2-3µl $MgCl_2$ (50mM), 1.25-5µl dNTPs (10nM), water, and buffer)	Cluster
ITS1-4*	ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') ITS4 (5'-TCCTCCGCTTATTGATATGC-3')	-Initial denaturation: 3min 97°C, Denaturation: 45s 95°C, Annealing: 45s 55°C, Extension: 45s 72°C, No. of cycles: 35, Final extension: 8min 72°C	'g,p'
		Initial denaturation: 5min 94°C, Denaturation: 5s 95°C, Annealing: 30s 54°C, Extension: 1min 72°C, No. of cycles: 35, Final extension: 10min 72°C	'c'
		Initial denaturation: 2min 95°C, Denaturation: 5s 95°C, Annealing: 15s 55°C, Extension: 10s 72°C, No. of cycles: 40, Final extension: 8min 72°C	'l'
		Initial denaturation: 4min 30s 95°C, Denaturation: 30s 95°C, Annealing: 30s 48°C, Extension: 1min 72°C, No. of cycles: 40, Final extension: 3min 72°C	'a,b,m,r,x,y,z', 'h,j,o'
		Initial denaturation: 15min 95°C, Denaturation: 1min 95°C, Annealing: 30sec 56°C, Extension 1min 72°C, No. Of cycles: 3, Final extension: 5min 72°C	'w'
	ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') ITS2 (5'-CCTCCGCTTATTGATATGCTTAGG-3')	Initial denaturation: 15min 95°C, Denaturation: 1min 95°C, Annealing: 30sec 56°C, Extension 1min 72°C, No. Of cycles: 39, Final extension: 5min 72°C	'w'
	SR6R (5'-AAGTATAAGTCGTAACAAGG-3') LR1 (5'-GGTTGGTTTCTTTCCT-3')	Initial denaturation: 3min 97°C, Denaturation: 35s 94°C, Annealing: 45s 50°C, Extension: 45s 72°C, No. of cycles: 30, Final extension: 7min 72°C	'g,p', 'i,þ'
	ITS2 (5-GCTGCGTTCTTCATCGATGC-3') ITS4 (5-TCCTCCGCTTATTGATATGC-3')	Initial denaturation: 3min 95°C, Denaturation: 40s 95°C, Annealing: 40s 51°C, Extension: 40s 72°C, No. of cycles: 35, Final extension: 7min 72°C	'h,j,o','e'
	ITS3 (5'-GCATCGATGAAGAACGCAGC -3') ITS4 (5'-TCCTCCGCTTATTGATATGC-3')	Initial denaturation: 2min 95°C, Denaturation: 30s 95°C, Annealing: 30s 55°C, Extension: 30s 72°C, No. of cycles: 35, Final extension: 5min 72°C	'q'
		Initial denaturation: 15min 95°C, Denaturation: 1min 95°C, Annealing: 30sec 56°C, Extension 1min 72°C, No. Of cycles: 39, Final extension: 5min 72°C	'w'
Barcoding*	AL33F/EF1-1018F (5' GAYTTCATCAAGAACATGAT 3') AL33R/ EF1-1620R (5' GACGTTGAADCCRACRTTGTC 3')	Initial denaturation: 5min 94°C, Denaturation: 50s 94°C, Annealing: 50s 48°C, Extension: 50s 72°C, No. of cycles: 40, Final extension: 7min 72°C	'g,p', 'i,þ'
		Initial denaturation: 15min 95°C, Denaturation: 1min 95°C, Annealing: 30sec 56°C, Extension 1min 72°C, No. Of cycles: 39, Final extension: 5min 72°C	'w'
	RPBF (5' GAYGAYCGKGAYCAYTTCGG 3') RPBR (5' CCCATRGCYTGYTTRCCCAT 3')	Initial denaturation: 5min 94°C, Denaturation: 30s 94°C, Annealing: 45s 50°C, Extension: 90s 50°C, No. of cycles: 35, Final extension: 5min 72°C	'g,p', 'i,þ'
Yeasts (D1/D2)	NL1 (5'- GCATATCAATAAGCGGAGGAAAAG –3') NL4 (5'– GGTCCGTGTTTCAAGACGG –3')	Initial denaturation: 5min 94°C, Denaturation: 5s 95°C, Annealing: 30s 54°C, Extension: 1min 72°C, No. of cycles: 35, Final extension: 10min 72°C	'c'
		Initial denaturation: 15min 95°C, Denaturation: 1min 95°C, Annealing: 30sec 56°C, Extension 1min 72°C, No. Of cycles: 39, Final extension: 5min 72°C	'w'
		Initial denaturation: 2min 95°C, Denaturation: 45s 95°C, Annealing: 30s 52°C, Extension: 2min 72°C, No. of cycles: 30, Final extension: 4min 72°C	'n', 'i,þ'

Cladosporium	ACT-512F (5'-ATGTGCAAGGCCGGTTTCGC-3') ACT-738R (5'-TACGAGTCCTTCTGGCCCAT-3')	Initial denaturation: 3min 94°C, Denaturation:30s 94°C, Annealing: 30s 55°C, Extension: 5min 72°C, No. of cycles: 7, Denaturation:30s 94°C, Annealing: 30s 53°C, Extension: 45s 72°C, No. of cycles: 25, Final extension: 5min 72°C	'n'
Aspergillus spp. and *Penicillium* spp.	Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3')	Initial denaturation: 5min 94°C, Denaturation: 5s 95°C, Annealing: 30s 60°C, Extension: 1min 72°C, No. of cycles: 35, Final extension: 10min 72°C	'c'
		Initial denaturation: 15min 95°C, Denaturation: 1min 95°C, Annealing: 30sec 56°C, Extension 1min 72°C, No. Of cycles: 39, Final extension: 5min 72°C	'w'
		Initial denaturation: 2min 95°C, Denaturation: 45s 95°C, Annealing: 45s 60°C, Extension: 90s 72°C, No. of cycles: 30, Final extension: 4min 72°C	'n'
Fusarium spp.	EF1 (5'–ATGGGTAAGGA(A/G)GACAAGAC–3') EF2 (5'–GGA(G/A)GTACCAGT(G/C)ATCATGTT–3')	Initial denaturation: 2min 94°C, Denaturation:40s 94°C, Annealing: 40s 60°C, Extension: 2min 72°C, No. of cycles: 8 (each cycle 1°C lower T), Denaturation:45s 94°C, Annealing: 90s 53°C, Extension: 2min 72°C, No. of cycles: 36, Final extension: 10min 72°C	'n'
black yeasts and other moulds*	ITS4 (5'-TCCTCCGCTTATTGATATGC-3) ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3')	Initial denaturation: 1min 94°C, Denaturation: 35s 94°C, Annealing: 53s 55°C, Extension: 30s 72°C, No. of cycles: 30 (each cycle 1°C lower T), Final extension:10 min 72°C	'n'

*Reviewed *in* Hoang MTV, Irinyi L, Chen SCA, Sorrell TC, the ISHAM Barcoding of Medical Fungi Working Group, Meyer W (2019). Dual DNA barcoding for the molecular identification of the agents of invasive fungal infections. Frontiers in Microbiology.10: article number 1647. http://doi.org/10.3389/fmicb.2019.01647

Table S9: Details on Identifications per laboratory cluster – Materials and Methods

Region	Cluster	Primary identification approach	Secondary identification approach	Tertiary Identification approach	DNA Extraction	Quality control
Southwest Europe	a,b,m,r, x,y,z	Macro and Microscopic analysis	VITEK 2 (Biomérieux) (for yeasts)	Molecular Identification with ITS2-ITS4 for moulds	PCR Template Preparation Kit (Roche Diagnostics GmbH)	ISO 15189 accredited
Northwest Europe	c	Macro and Microscopic analysis	MALDI-ToF (Bruker) (for yeast and filamentous fungi)	Molecular Identification with ITS2-ITS4, D1-D2 and Bt2a-Bt2b, if Maldi biotyper Logscore <1.9	QIAamp DNA Mini kit	ISO 15189 accredited
Mediterranean	d	Macro and Microscopic analysis	MALDI-ToF (Bruker) (for yeast and filamentous fungi)	n.a.	n.a.	JCI Hospital Accredited, API (for External Quality Assessment)
Mediterranean	e	Macro and Microscopic analysis	MALDI-ToF MS (Biomérieux)	ITS2-ITS4	Qiagen DNA minikit	ISO 15189 accredited
Black Sea	f	Macro and Microscopic analysis	MALDI-ToF MS (Bruker) (for yeasts and common filamentous fungi)	Molecular Identification with ITS2-ITS4 for moulds	n.a.	ISO 15189 accredited
Sydney, Australia	g,p	MALDI-ToF MS for yeast (Bruker)	ITS1/2, elongation factor 1 alpha(TEF1α) and RNA polymerase II gene (RPB2)	n.a.	phenol:chloroform:isoaml alcohol (25:24:1)	Speciation specialist
Mediterranean	h,j,o	Macro and Microscopic analysis	API ID32C assimilation kit for yeasts (bioMérieux), MALDI-ToF VITEK SYSTEM, IVD, v. 3.2, (Biomérieux)	Molecular Identification With ITS2-ITS4 for moulds	QIAGEN DNeasy Power Soil Pro Kit	UK NEQAS external quality programme, JCI HospC8:H12ital Accredited (site j)
Mediterranean	i,Þ	Macro and Microscopic analysis	MALDI-ToF MS (Bruker) (for yeasts and common filamentous fungi)	Molecular Identification sequencing of ITS Barcoding region (for yeasts not in the MALDI-ToF MS database & rare moulds	Phenol/chloroform	ISO 15189 accredited
Mediterranean	k	Macro and Microscopic analysis	MALDI-ToF (Bruker) With two data bases: Bruker & MSI	Molecular Identification with ITS 1-5-8, ITS2	Phenol/chloroform	ISO 17025 accredited
Northwest Europe	l	Macro and Microscopic analysis	Candida ID agar (94382) for yeasts	Molecular Identification with ITS1-ITS4 for moulds	QIAGEN DNeasy PowerSoil Pro Kit for moulds and Phenol/Chloroform for Yeasts	Reference strains
Mediterranean	n	Macro and Microscopic analysis	Molecular Identification: NL1 & NL4 (yeasts), ACT-512F & ACT-738R (*Cladosporium*), Bt2b & Bt2a (*Aspergillus*, *Penicillium*), EF1 & EF2 (*Fusarium*), ITS4-ITS5 (for black yeasts & other moulds)	n.a.	Phenol/chloroform and, PrepMan Ultra reagent	Speciation specialist
Northwest Europe	q	Macro and Microscopic analysis	Molecular Identification with ITS3-ITS4 (White et al., 1990)	n.a.	QIAGEN DNeasy PowerSoil Pro Kit	Identification of reference strains (Genebank sequence database).
Northwest Europe	s	Macro and Microscopic analysis	MALDI-ToF MS (Bruker) (for yeasts)	n.a.	n.a.	ISO 15189 accredited
Mediterranean	t	Macro and Microscopic analysis	Vitek and Auxacolor (for yeasts)	n.a.	n.a.	UK NEQAS external quality programme
Mediterranean	u	Macro and Microscopic analysis	MALDI-ToF MS (Bruker) (for yeasts and filamentous fungi)	n.a.	n.a.	UK NEQAS external quality programme
Mediterranean	v	Macro and Microscopic analysis	MALDI-ToF MS (for yeasts and filamentous fungi)	n.a.	n.a.	UK NEQAS external quality programme
Mediterranean	w	Macro and Microscopic analysis	MALDI-ToF MS (for yeasts and filamentous fungi)	Molecular Identification when Maldi biotyper Logscore <1.9 with ITS1-ITS2, ITS3-ITS4, ITS1-ITS4, Translation elongation factor (TEF 1-a), Beta-tubuline2, D1D2 regions (LSU)	QIAGEN Eazy One	ISO 15189 accredited for Secondary and Tertiary approach

Chapter 7

Untreated sewage contamination of beach sand from a leaking underground sewage system - An episode of skin rash was experienced by 30 people at a beach.

Brandão, J., Albergaria, I., Albuquerque, J., José, S., Grossinho, J., Ferreira, F. C., Raposo, A., Rodrigues, R., Silva, C., Jordao, L., Sousa, M., Rebelo, M. H., Veríssimo, C., Sabino, R., Amaro, T., Cardoso, F., Patrão-Costa, M., Solo-Gabriele, H.

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Untreated sewage contamination of beach sand from a leaking underground sewage system

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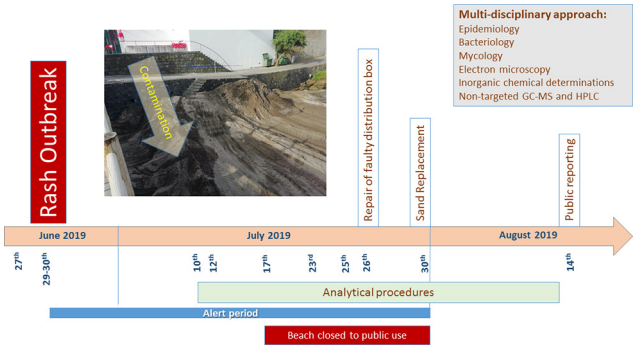
HIGHLIGHTS

- An episode of skin rash was experience by 30 people at a beach.
- Analysis of the sand revealed a substance compatible with NaOCl concomitant high levels of faecal indicator organisms.
- Sodium-hypochlorite was used for cleaning and disinfection of toilet facilities.
- A leakage in the sewage system was found to have been the cause of the outbreak.

GRAPHICAL ABSTRACT



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ABSTRACT

Thirty people (mostly children) experienced an episode of skin rash days after a sand sifting beach operation at Porto Pim Beach in Faial, Azores during June 2019. An environmental and epidemiologic investigation was conducted to identify the cause of the outbreak of skin rash. The epidemiologic investigation found that some of the patients experiencing symptoms had never entered the beach water. During the pollution period and throughout the epidemiologic investigation, faecal indicator bacteria levels (94 CFU/100 ml for intestinal enterococci and 61 CFU/100 ml for *Escherichia coli*) in water remained under the limits used for the ninety-five percentile calculation of an Excellent coastal and transitional bathing water defined in the Portuguese Legislation (100 CFU/100 ml for intestinal enterococci and 250 CFU/100 ml for *Escherichia coli*). Thus sand contact was considered as a likely primary exposure route. Sand microbiological analysis for faecal indicator organisms and electron microscopy strongly suggested faecal contamination. Chemical analysis of the sand also revealed a concomitant substance compatible with sodium-hypochlorite as analysed using gas chromatography and subsequently confirmed by free chlorine analysis. Inspection of the toilet facilities and sewage disposal system revealed a leaking sewage distribution box. Collectively, results suggest that the cause of the outbreak was the leaking underground sewage distribution box that serviced the beach toilet facilities (40 m from beach), where sodium-hypochlorite was used for cleaning and disinfection. This sewage then contaminated the surficial sands to which beach goers

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were exposed. Chlorine being an irritant substance, was believed to have been the cause of the symptoms given the sudden presentation and dissipation of skin rashes. No gastro-intestinal illness was reported during this episode and during the following 30 days. Like water, beach sand should also be monitored for safety, especially for areas serviced by aged infrastructure.

1. Introduction

Beach sand is known to harbour microbes and other pollutants. The source of pollutants can come from humans (Elmir et al., 2007; Elmir et al., 2009; Hughes et al., 2017; Torres-Bejarano et al., 2018), or other animals such as seabirds (Lu et al., 2011; Alm et al., 2018) and dogs (Wright et al., 2009). As a result of its ability to accumulate pollutants, beach sand has long been considered a possible source of water pollution by run-off and tide retraction (Vogel et al., 2016; Weiskerger et al., 2019; Whitman et al., 2014; Feng et al., 2015). Microorganisms in beach sand can also affect beach goers by direct contact with skin and incidental ingestion or inhalation, which may result in exposure to pollution, including microorganisms known to cause infections and exacerbate allergies (Sabino et al., 2014; WHO, 2003; Fleisher et al., 2010; Shibata and Solo-Gabriele, 2012). These include many fungi and bacteria, possibly viruses, protozoa, and some helminths in tropical beaches (Sabino et al., 2014; Shah et al., 2011).

Epidemiologic studies that relate human illness to beach sand quality are few (Lamparelli et al., 2015; Praveena et al., 2016; Bonilla et al., 2007; Esiobu et al., 2013). Heaney et al. (2012) demonstrated a positive relationship between sand contact activities and enteric illness as a function of concentrations of faecal microbial organisms in beach sand. Sabino et al. (2011) based upon the distribution of Faecal Indicator Bacteria (FIB) found in the environment recommends maximum allowable values of 25 CFU/g for *E. coli* and 10 CFU/g for enterococci.

Although microbial contaminants have been reported in beach sand and studies have linked human health to beach sand quality (Solo-Gabriele et al., 2016), no report to date has been published of a beach outbreak of human illness, that has been attributed solely to beach sand and excludes water as a possible exposure route. An outbeak is defined here as the occurrence of disease cases in excess of normal expectancy (WHO, 2020).

The objective of the current study was to identify the cause of the June 2019 outbreak at Porto Pim Beach in Faial, Azores. The approach included epidemiologic and environmental investigations for which beach sand was suspected to have been associated with skin rash. To facilitate the investigation, the sand was analysed for both microbiological and chemical contaminants. Microbiological analysis included measurements of faecal indicator bacteria, fungi, and the evaluation of sand grain colonization by microbial communities, such as biofilms, through scanning electron microscopy. Due to the shortness of the skin symptoms which suggested a contact dermatitis, the chemical analyses were extensive and included measurements of both organic and inorganic chemicals.

2. Materials and methods

2.1. Outbreak detection

On the 27th of June, a regular sifting action took place up to 4 cm deep in the beach sand of Porto Pim in Faial, Azores. The purpose of the sifting was to remove debris and litter on the sand surface using a vehicle with a vibrating sieve in the back and dragging residual amounts of sand as it moved.

During the weekend of 29th to 30th of June of 2019, 29 children and one adult at the beach suffered macular erythematous rashes with concomitant itching in areas of their bodies that were not covered by bathing garments. Either the victims or respective adults in charge individually reported the cases to the lifeguards at the beach. Some of the cases were further followed by medical personnel, as described in "Section 2.2 Epidemiology". This study is based on data collected anonymously rendering the study compliant with the World Medical Association's Helsinki Declaration of 2013.

2.2. Epidemiology

The 29 affected children were between 18 months and 8 years of age (female/male ratio undetermined) and the adult was a male of 44 years of age. Following the onset of symptoms, some of the victims (exact number unknown) sought medical assistance at the Children's Health Unit of Horta and at the Emergency Room of Horta Hospital, where macular erythematous pruritic rash diagnoses were confirmed.

Most cases resolved spontaneously within a few hours therefore requiring no excessive treatment. This was reported verbally by the victims or their representative adults in charge to the lifeguard and local health authorities, following instructions upon reporting of the onset of the symptoms.

The following seven cases were further registered in general practice appointments: a six-year old girl with macular erythematous pruritic rash following contact with the sand at the beach; a 44 year old immunocompetent male who developed pruritic rash following contact with sand, three girls (18 months, 5, and 8 years old) and two boys (2 and 7 years old) also with macular erythematous pruritic rash following contact with sand, also resolving spontaneously hours thereafter.

During the 30 days following the outbreak there were no new reports to the local hospitals (Alert period in Fig. 1), neither as skin problems nor for gastro-intestinal illnesses, as monitored by the public health authorities.

The area associated with this episode is identified as 'Area associated with the outbreak' in Fig. 2, and that beach usually will have been visited by a total of just under one hundred people during the weekend of the outbreak, as estimated by Porto Pim beach managers.

2.3. Sampling

Dry (supratidal) sand samples (up to 10 cm deep) were collected aseptically into a sterile plastic bag, labelled, and transported in a cooler to the lab as described in Sabino et al. (2011). The 1st campaign (10th July 2019) consisted of two samples, a grab sample 'A', following a request of the beach management, and a composite sample 'B' of the locations of samples 6, 7 and 8, to represent that section of the beach, following recommended procedures (Sabino et al., 2011). The 2nd campaign (23rd July 2019) included 10 grab samples identified as '1' through '10' in Fig. 2. Samples A and 2 are identical in location, 13 days apart. Weather conditions during the days of sampling were between 20.0 and 22.2 °C for air temperature, partially cloudy, dry and with relatively low wind speeds (between 11 and 24.1 kmh^{-1}).

2.4. Bacterial faecal indicators

Fifty grams of sand were extracted with 500 ml of sterile distilled water, rotating top-bottom for 30 min at 100 rpm and analysed for FIB including total coliforms/*Escherichia coli* (*E. coli*) and enterococci, using Colilert™ and Enterolert™ with Quanti-Tray (IDEXX Laboratories, Maine, USA), respectively. From positive wells identified in the trays, the FIB were enumerated in units of most probable number (MPN) per gram of sand (original sample - not dry weight).

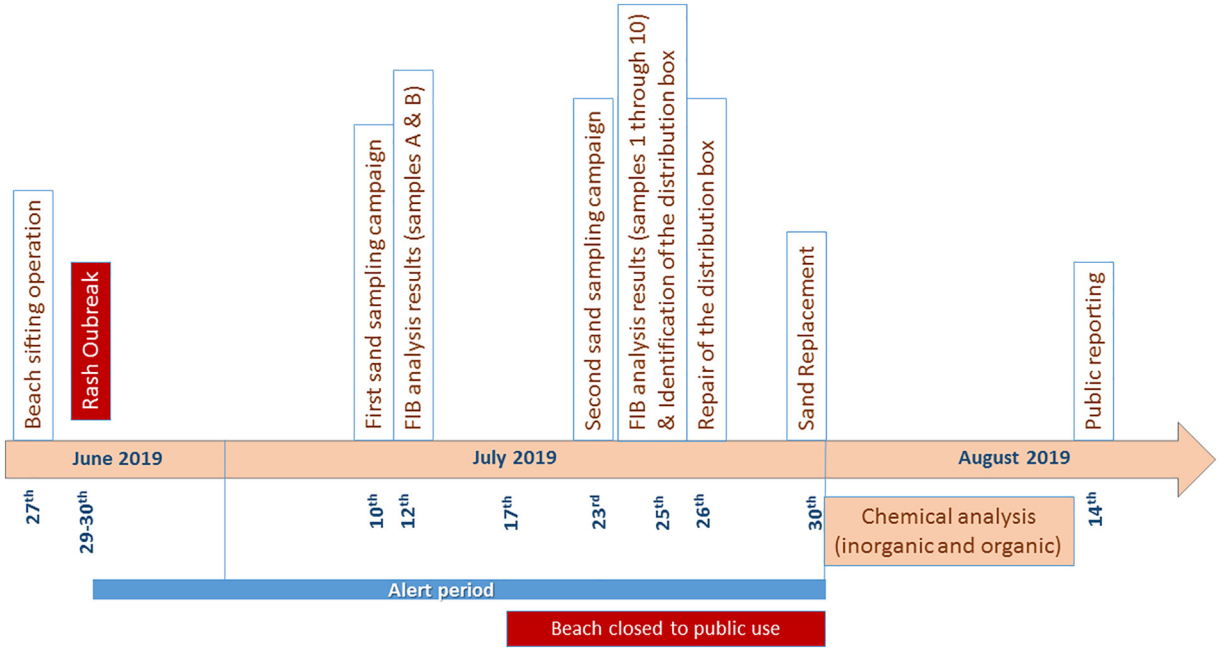

Fig. 1. Timeline of events related to the detection and resolution of the outbreak.

Fig. 2. Areal view of Porto Pim beach (latitude 38° 31′ 24″ N, longitude 28° 37′ 33″ W) showing the location of the sampling points of the second sampling campaign and area associated with the outbreak. Sample A of the first campaign took place in the same location as 2 and sample B is a composite of the locations of samples 6, 7 and 8. Base map from Google Earth.

2.5. Mycology

Forty grams of sand were extracted with 40 ml of sterile distilled water by orbital shaking for 30 min at 100 rpm and the extract was then plated (0.2 ml) in triplicates per media (Sabouraud 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 ± 0.5 °C. Colonies were counted and the results were given in colony forming units (CFU) per gram of sand (equivalent), as mean number of each triplicate. The predominant species were isolated and identified posteriorly.

2.6. Electron microscopy

Sand samples were prepared for scanning electron microscopy in topographic mode as previously described (Bandeira et al., 2014). Representative micrographs of samples A, B (1st campaign) and 2 (2nd campaign) are shown in Fig. 3A, B and C, respectively.

2.7. Chemical analysis

Only samples A, B and 3 to 6 were processed for both inorganic and organic analysis. Inorganic analysis included samples 1, 2 and 10, and comprised of pH, conductivity, ammonia, nitrate, nitrite and free chlorine. Samples 1–10 were not tested for ammonia, nitrate, nitrite because the results of A and B of these parameters were below detection limits. Organic analysis focused on the measurements of the following polycyclic aromatic hydrocarbons (PAHs): benzo(*a*)pyrene (B(*a*)P), benzo(*k*)fluoranthene (B(*k*)F), benzo(*b*)fluoranthene (B(*b*)F), benzo(*g*,*h*,*i*)perylene (B(*g*,*h*,*i*)P), indeno(1,2,3-c,d)pyrene (I(1,2,3-c,d)P), fluoranthene (Flu), pyrene (Pyr), and benzo[*a*]anthracene (B(*a*)A). For inorganic analyses, 5 g of air-dried sample was extracted by shaking for 30 min in 200 ml of deionized water. Samples were analysed using ISO 7150/1 standard for ammonia (International Standard ISO 7150/1, 1984) and the standard methods for water samples for the remaining parameters (Baird et al., 2017). For organic analysis, 2 g of sample were extracted by ultrasonic bath for 30 min in 15 ml. of organic solvent (DCM/MeOH 2:1) (Šrut et al., 2011). Extracts were analysed using high

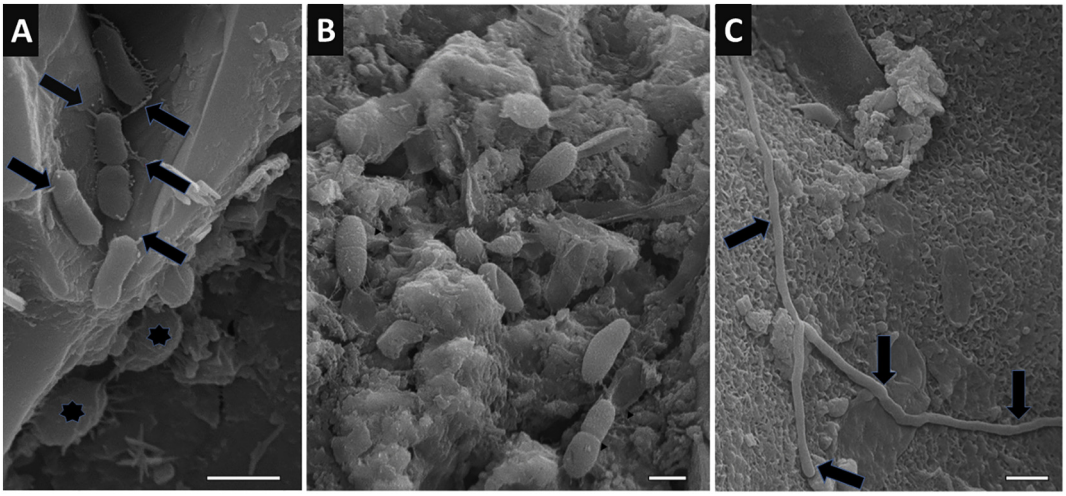

Fig. 3. Scanning electron micrographs of sand samples A (3.A), B (3.B) and 2 (3.C) showing colonisation by bacteria and/or fungi. Yeast (asterisks in A), hyphae (arrows in C) and bacterial fimbriae (arrows in A) are highlighted in the micrographs. Scale bar = 1 μm.

pressure liquid chromatography with fluorescence detector (HPLC-FLD) (García-Falcón et al., 2004) and gas chromatography–mass spectrometry (GC–MS). GC–MS was performed in both targeted and non-targeted mode. Details are provided in the supplemental text.

3. Results

3.1. Bacterial faecal indicators

The first sampling campaign yielded levels of FIB above quantification limits (>201 MPN/g) for all three FIB evaluated. These results were significantly above the maximum reference values as recommended by Brandão et al. (2002) of 100 MPN/g for coliforms, 20 MPN/g for *E. coli*, and 20 MPN/g for enterococci. Elevated levels were also detected in samples collected during the second sampling campaign, in particular for sample 2 where both coliforms and enterococci measured at values >201 MPN/g. Sample 3 also showed elevated levels of coliforms (>201 MPN/g) and sample 4 was elevated for enterococci (145 MPN/g) (Table 1). Overall, the trend was for the concentration of the FIB to decrease with distance from the toilet facilities.

3.2. Mycology

The first campaign yielded 100 CFU/g and no yeasts for sample A. Fifty-percent of the colonies were identified as *Fusarium* sp. and the remaining 50% were identified as varied filamentous fungi, including black mould. Sample B yielded 109 CFU/g of unidentified varied filamentous fungi, including black mould, and 88 CFU/g of *Meyerozyma guilliermondii* plus 2 CFU/g of *Candida tropicalis*. The second set of samples yielded the results shown in Table 1, with sample 6 being mainly composed of *Fusarium* sp. and sample 7 of *Aspergillus* section *Circundati*. Sample 3 was very rich in *Rhodotorula* sp.

3.3. Electron microscopy

No complex biofilms were seen in the samples. The morphologic analysis of the different microorganisms (bacteria and fungi) present in sand samples was in agreement with the bacterial faecal indicator data. Rod shape bacteria with or without *fimbriae* were observed in all samples (Fig. 3A and B). Fungi and yeasts were also abundant as shown in Fig. 3A, but hyphae were only observed in sample 2 (Fig. 3C). Structures consistent with mature biofilms were not observed although incipient associations could be identified. In Fig. 3A the development of *fimbriae* for bacterial adherence to the sand surface and to other bacteria was observed. Overall the microorganism structures appear to be consistent with early stage biofilms.

3.4. Chemistry

The sand of Porto Pim is alkaline consisting of a mixture of silica, limestone (calcium carbonate), possibly clay (complexed minerals) and visible volcanic matter. Its pH varied between 9.0 and 9.5, as measured at 23 °C.

Most of the samples contained traces of PAHs (Table 2), which were probably due to the partially volcanic nature of the sand (expressed with the symbol ✔). Non-targeted GC–MS analysis in the 6 samples tested found chromatographic peaks of mass to charge ratio consistent with sodium hypochlorite (NaOCl). The second sampling campaign revealed higher concentrations of this compound, which suggests an on-going contamination increasing in intensity, and consistently decreases along with the distance from the pollution source. Data from inorganic analysis were consistent with these results. Levels of ammonium, nitrate and nitrite were below quantification limits whereas samples A and 1 through 4 revealed the presence of free chlorine, which is non detectable in site 10 (used to assess an eventual environmental chlorine presence).

3.5. Outbreak control measures

Based on the bacteriology results, the local team closed the beach to public use (on the 17th of July) and went looking for possible sources of faecal contamination and found a degraded distribution box between the septic tank from public toilet facilities and the subsurface drain system. The spatial disposition of the infrastructure relative to the beach is shown in Fig. 4. The box bottom was rebuilt and the sides were waterproofed. Fig. 4 (Panel B) shows also the first stage of the tank restoration, which was followed by waterproofing the sidewalls. Contaminated sand (80 m^3) was removed to a 50 cm depth and replaced by an uncontaminated batch (Fig. 4, panel C2). The beach was reopened for public use on the 31st and the sand was checked regularly for faecal and environmental contamination until the end of the bathing season, with results within the national limits described in Brandão et al. (2002).

4. Discussion

To the authors' knowledge, this is the first beach outbreak associated exclusively with sand. The cause of the rash may have been sodium hypochlorite (NaOCl, bleach) but confirmation is not possible. The European Chemical Agency states that the human skin's threshold for symptoms

Table 1
Analytical results (MPN/g for FIB and CFU/g for fungi, yeasts and dermatophytes). Results are highlighted when they exceed the maximum reference values according to Brandão et al. (2002).

	Coliforms (100)*	*E. coli* (20)*	Enterococci (20)*	Filamentous fungi (560)*	Yeast (60)*	Dermatophytes (15)*
1st campaign (10th July 2019)						
Sample A	>201	>201	>201	100 (50 *Fusarium* sp)	<1.0	<1.0
Sample B	29	<1.0	9	109	90 (88 *M. guilliermondii* + 2 *C. tropicalis*)	<1.0
2nd campaign (23rd July 2019)						
Sample 1	2	2	4	84	<1.0	<1.0
Sample 2	>201	<1.0	>201	385	<1.0	<1.0
Sample 3	>201	<1.0	6	85	65 (64 *Rhodotorula* sp.)	<1.0
Sample 4	33	<1.0	145	125	<1.0	<1.0
Sample 5	23	<1.0	<1.0	107	<1.0	<1.0
Sample 6	<1.0	<1.0	<1.0	190 (152 *Fusarium* sp.)	<1.0	<1.0
Sample 7	13	<1.0	1	183 (165 *Aspergillus* section *Circundati*)	2	<1.0
Sample 8	9	<1.0	<1.0	55	<1.0	<1.0
Sample 9	2	<1.0	13	5	2 (*Rhodotorula* sp.)	<1.0
Sample 10	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0

'MPN' = Maximum Probable Number, 'CFU' = Colony Forming Unit. *Maximum reference values per gram of sand.

Table 2
Analytical results for organic and inorganic chemical analysis and GC–MS peaks compatible with NaOCl.

Samples	PAH								Chromatographic peak area for NaOCl*	Inorganic analysis						
	B(a)P	B(k)F	B(b)F	B(g,h,i)P	I(1,2,3-c,d)P	Flu	Pyr	B(a)A		pH	Conductivity µS/cm	Ammonium (mg/L)	Nitrate (mg/L)	Nitrite (mg/L)	Free chlorine (mg/L)
1st campaign (10 July 2019)																
Sample A	✔	–	–	✔	✔	–	–	–	463	9.2	75	<LQ	<LQ	<LQ	0.06
Sample B	✔	✔	✔	✔	✔	✔	✔	–	559	9.0	120	<LQ	<LQ	<LQ	<LQ
2nd campaign (23 July 2019)																
Sample 3	✔	✔	✔	✔	✔	–	–	–	2323	9.2	130	N/A	N/A	N/A	0.09
Sample 4	–	–	–	–	–	–	–	–	2610	9.2	128	N/A	N/A	N/A	0.07
Sample 5	–	–	–	–	–	–	–	–	1166	9.0	108	N/A	N/A	N/A	0.06
Sample 6	✔	–	–	–	–	–	–	–	550	9.1	120	N/A	N/A	N/A	0.12
Sample 10	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	9.5	73	N/A	N/A	N/A	<LQ

'✔' = detected (just over the detection limits 0.00200 µg for B(*a*)P and 0.00400 µg for all other PAHs), '–' = undetected, '<LQ' = under the limit of quantification (LQ), "N/A" = not analysed.
*Analytical results for organic compounds by GC–MS corresponding to a mass to charge ratio of 69.0 which is consistent with the detection of sodium hypochlorite.

Fig. 4. On the top left (A) - the lid of the distribution box shown by the red circle. On the top right (B) – the inside of the distribution box after partial recovery (bottom) and before fully sealing the sidewalls. At the bottom, C1 illustrates the location of the distribution box relative to beach access. C2 shows the mechanical removal of the contaminated sand, as delineated by the analytical results on FIB until 50 cm deep (80 m^3 in total), including the location of the 2nd campaign sampling points 1 through 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

due to contact with NaOCl lies at 3.9% (The European Chemical Agency, n. d.; Kozol et al., 1988). The symptoms, however, were compatible with a topical irritant like sodium hypochlorite (Punjataewakupt et al., 2019; Chia Shi Zhe et al., 2016), which was confirmed to be used in surface and toilet cleaning. Very likely, the number of days between the sampling and analytical procedures resulted in a significant reduction of the original concentrations. Although sunlight, heat and the presence of metals would degrade the chlorine residual (Lantagne et al., 2011), the calcium carbonate composition of the sand and the high pH could have contributed towards the persistence of the NaOCl. These later factors could have contributed to the patients' symptoms.

The fungal analysis revealed the presence of *Rhodotorula*, which does not indicate any specific source of skin irritant but is compatible with faecal contamination (Khatib et al., 2001). Most of the other species found can be associated with either marine water or vegetable matter and do not cause symptoms compatible with this outbreak. The FIB have the highest counts in points 2–4 which is the area where *Rhodotorula* sp is at its highest concentration also. This is consistent with the expected proximity to the pollution source, the area in which the NaOCl is also at its highest concentration (sampling location 4).

Aged facilities are the cause of many sewage-spill episodes (Su et al., 2020; Barreras et al., 2019). The identified origin was an underground source of sewage spillage that ran off into sand directly below, contaminating it (see Fig. 4 for configuration). Septic tank systems have been identified as sources of groundwater contamination (Ferrer et al., 2020; Jangam and Pujari, 2019; Sinigalliano et al., 2007). Verhougstraete et al. (2015) found that septic tanks were the cause of elevated faecal bacteria levels in rivers within 64 watersheds. Although contamination by septic tanks is most commonly observed in water, the unique feature about this outbreak is that the contamination impacted beach sand. The impact to sand was due to the configuration of the toilet facilities relative to the beach where the distribution box was at an elevation above the beach sand allowing for sewage to impact surficial beach sands. The sifting of the sand shortly before the outbreak could have exposed impacted sands below the surface, where exposure with humans was more likely.

It is possible that the contamination from the sewage distribution box, that impacted the sand also eventually impacted the water. However, levels in water measured through weekly routine monitoring were below detection limits for the sampling done immediately before the episode (24th of June). Immediately after the episode (2nd of July) levels detected in the water were at 94 CFU/100 ml for intestinal enterococci and 61 CFU/100 ml for *Escherichia coli*, which were still under the limits used for the ninety-five percentile calculation of an Excellent coastal and transitional bathing water defined in the Portuguese Legislation (which are 100 CFU/100 ml for intestinal enterococci and 250 CFU/100 ml for *Escherichia coli*). Subsequent sampling on the 9th and 15th of July showed that

water quality returned to below detection limits for FIB, as compared to sampling conducted earlier. The increase in FIB observed for the water on the 2nd of July may have been from the same underground source of sewage that contaminated the sand.

Although the levels of FIB in the water increased immediately after the episode, the levels were not above advisory levels. Thus, the source of the outbreak is believed to be the NaOCl in the sand, primarily because the symptoms were not gastrointestinal and disappeared in a few hours which is not typical of a waterborne illness (Leonard et al., 2018). Additionally, some individuals experiencing symptoms had no contact with the water. Furthermore, this case did not particularly require confirmation of human sewage contamination with a microbial source tracking method (MST) because NaOCl does not exist naturally in beach sand. However, during a microbial outbreak analysis, source tracking approaches are strongly advised in order to help identify the source of faecal pollution (Nguyen et al., 2018; Teaf et al., 2018; Kirs et al., 2017). In this case the source was identified as an underground sewage distribution box that services the beach toilet facilities, which in turn did not require additional analysis through MST.

Some of the superficial sand may have been dragged by the sifting vehicle from points 1–3 into 4, 5 and 6. In 2006, a sewage spill that contaminated beach sand between Manhattan Beach and Palos Verdes (about 18 km), in Lower California (USA), resulted in the use of bleach to neutralise pathogens. A berm was also built to avoid sewage reaching the water (Environmental News Network, 2006). It is unknown whether the bleach used to disinfect the sand affected the beach goers afterwards. In the current study the chlorine concentration in nearshore waters was undetermined but no cases of concomitant gastro-intestinal illness in those that bathed and suffered a rash were reported to the local health authorities but also not by other bathers during the weekend for the following 30 days. Contact with sand as opposed to the water is thus believed to have been the sole cause of the rashes. This episode demonstrates that sand pollution alone can be harmful to beach users.

5. Conclusions

In addition to inspecting and managing beaches, beach sand should be regulated and monitored for microbiological constituents in a similar fashion as water, especially in areas serviced by aged infrastructure. Microbiological monitoring would have detected the contamination given that FIB levels were high. If such monitoring were in place, the outbreak could have been averted due to the high levels of FIB in sand caused by the sewage contamination. This was the lesson learned by the local team, which is implementing sand analysis regularly throughout beaches of their administrative jurisdiction.

CRediT authorship contribution statement

J. Brandão: Conceptualization, Writing - review & editing, Formal analysis, Investigation. **I. Albergaria:** Investigation. **J. Albuquerque:** Investigation. **S. José:** Investigation. **J. Grossinho:** Investigation. **F.C. Ferreira:** Investigation. **A. Raposo:** Investigation. **R. Rodrigues:** Investigation. **C. Silva:** Investigation. **L. Jordao:** Investigation. **M. Sousa:** Investigation. **M.H. Rebelo:** Methodology. **C. Veríssimo:** Investigation. **R. Sabino:** Investigation. **T. Amaro:** Writing - review & editing. **F. Cardoso:** Project administration. **M. Patrão-Costa:** Project administration. **H. Solo-Gabriele:** Conceptualization, Writing - review & editing.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.140237.

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Supplemental Text for

Untreated sewage contamination of beach sand from a leaking underground sewage system

Details of Chemical Analyses

1. Standards Preparation

A mix of fluoranthene (Flu), pyrene (Pyr), benzo(a)anthracene (B[a]A) was prepared to obtain a 0.1 mg/L in acetonitrile; A mix of benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[g,h,i]-perylene and indeno[1,2,3-c,d]pyrene was prepared to obtain a 0.1 mg/L in acetonitrile; Benzo[a]pyrene was prepared to obtain a 0.05 mg/L in acetonitrile

2. Methods

Chromatography, high pressure liquid chromatography (HPLC) [4] and gas chromatography–mass spectrometry (GC-MS) were used for organic compounds. Molecular absorption spectroscopy (MAS) method was used for inorganic parameters. Ammonium quantification was based on ISO 7150/1 – 1984 (E) [1] and Nitrate and nitrite quantifications were based on the *Standard Methods* [2].

3. Preparation of sand samples

Following Hazel Davidson's technique, once a subsample of sand was weighed out, some form of liquid was added to the sand to extract the required compound of interest. For inorganic compounds ultra-pure water was used and for organic a compatible extraction solvent was utilized. Samples were shaken, refluxed, or digested for specific periods of

time, then filtered or centrifuged, and the liquid extract analysed by an instrument specific for the analyte in question.

4. Inorganic Compounds

The dried sand was weighed out (5 g of sample), and 200 mL of pure water was added, followed by 30 min shaking at 100 rpm to dissolve most of the polar compounds. After shaking the samples were filtered. The filtrate was then subjected to ammonium and nitrate/nitrite analysis.

5. Organic Compounds

Dried sand was weighed out (2 g of sample) and 15 mL of DCM/MeOH (2:1) was added as an extraction solvent. Extraction was done in an ultrasonic bath at 25ºC for 30 min. Samples were evaporated to dryness under a gentle stream of nitrogen flow. Residue was dissolved in 1 mL of mobile phase for HPLC (ACN) or 1 mL of mobile phase for GC-MS (AcOet) injection [3].

6. Chromatographic Conditions

All HPLC-FLD were performed with Agilent 1100 equipment. The PAHs were detected by fluorescence detection (FLD). Analysis using the C18 column were carried out with acetonitrile. Wavelengths for excitation (280 nm) and emission (430 – 500 nm) were selected at their elution retention time for a 15.5 min run for B[k]F ,B[b]F, B[a]P, B[g,h,i]P and I[1,2,3-c,d]P.

A second wavelength program was used for quantifying fluoranthene, pyrene and benzo[a]anthracene for a 10 min run. Wavelengths used for Flu and Pyr were 280 nm

for excitation and 430 nm for emission. For B[a]A wavelengths used were 250 nm for excitation and 430 nm for emission.

All GC-MS was performed with a Varian 4000 gas chromatograph, coupled to a mass spectrometric detector. The samples were injected in the splitless mode at an injection temperature of 250 °C. The transfer line and ion source temperatures were at 280 °C and 200 °C. The column temperature was initially set to 50 °C for 1 min, raised to 70 °C at the rate of 20 °C/min, then to 170 °C at the rate of 10 °C/min, and finally to 190 °C at the rate of 2 °C/min; followed by holding at the final temperature for 32 min. Helium was used as a carrier gas at a constant flow rate of 1 mL/min. Signal detection was via mass spectrometry using the electron ionization (EI).

7. Reagents manufacturing information

HPLC grade, acetonitrile, ethylacetate (AcOet) and dichlormethane (DCM) were purchased from Merck. Methanol (MeOH) was purchased from Pestinorm. Ultrapure water was prepared by ultrafiltration with a Milli-Q water purification system from Millipore. Individual Polycyclic Aromatic hydrocarbons (PAHs) standards (fluoranthene, pyrene, benzo(a)anthracene, benzo(a)pyrene (B[a]P), benzo(b)fluoranthene (B[b]F), benzo(k)fluoranthene (B[k]F), benzo(g,h,i)perylene (B[g,h,i]P) and indeno[1,2,3-c,d]pyrene (I[1,2,3-c,d]P)) were purchased from Dr. Ehrenstorfer. Acetonitrile (Merck, HPLC grade) was used for preparing the solutions' standards.

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Chapter 8

Chapter 6 - Beach sand

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(This chapter presents only a draft version of the section on microbial quality of beach sand. The blank pages correspond to other sections that are not part of the subject of this thesis, and are therefore not presented, for confidentiality reasons since these guideline were not published at the time of submission of this thesis)

WHO GUIDELINES FOR RECREATIONAL WATER QUALITY

Version 6 – March 2021 for final edit

Contents

Foreword

To be added in final version

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Acronyms and abbreviations

AFRI	acute febrile respiratory illness
ALF	alert level framework
CFU	colony-forming unit
CSO	combined sewer overflow
EU	European Union
ELISA	enzyme-linked immunosorbent assay
FIO	faecal indicator organism
GI	Gastrointestinal
GDWQ	World Health Organization Guidelines for drinking-water quality
GV	guideline value
HAB	harmful algal and cyanobacterial bloom
HPLC	high-performance liquid chromatography
IRP	incident response plan
LC	liquid chromatography
LOAEL	Lowest-observed-adverse effect level
MPN	most probable number
MRSA	methicillin-resistant Staphylococcus aureus
MST	microbial source tracking
NOAEL	no observed adverse effect level
PAM	primary amoebic meningoencephalitis
PCR	polymerase chain reaction
QMRA	quantitative microbial risk assessment
qPCR	quantitative polymerase chain reaction
RWSP	Recreational Water Safety Plan
TCiW	Toxic cyanobacteria in water (WHO publication)
TP	total phosphorus
UK	United Kingdom
USA	United States of America
WHO	World Health Organization

Executive Summary

Scope

The WHO Guidelines for Recreational Water Quality (coastal water and freshwater) aim to protect public health by ensuring that recreational water quality is safely managed.

Use of coastal, esturine and freshwater recreational water environments has significant benefits for health and well-being, including rest, relaxation, exercise, cultural and religious practices and aesthetic pleasure. It also brings substantial local, regional and national economic benefits from tourism. However, recreational water

environments also contain potential hazards, which must be weighed against the benefits. The guidelines focus on water quality management for coastal and freshwater. Recreational water sites are also natural ecosystems that support a range of aquatic organisms including fish and shellfish, insects and birds some of which present nuisance or injury or possible health hazards to humans. Application of these guidelines should be complementary to measures for protection of aquatic ecosystems.

Treated swimming pools and spas as well as other recreational water hazards such as downing, sun, heat and cold, dangerous aquatic organisms are addressed in other WHO guidelines (refer to Table 1.2).

These guidelines are targeted primarily to national and local authorities and other entities with obligation to exercise due diligence relating to the safety of recreational water sites and many be implemented in conjunction with measure for envonmental protection of recreational water use sites.

The guidelines apply to the general population, unless otherwise mentioned participating in all types of recreational water use entailing direct water contact, inhalation of aerosols as well as beach use. Immunocompromised individual should seek medical advice on their individual ability to withstand the exposures to surface recreational waters. The guidelines;

- describe the current state of knowledge about the possible adverse health impacts of recreational use of coastal, estuarine and freshwater environments; and
- set out recommendations for setting national health-based targets, risk assessment and management approaches to identify, monitor and control these hazards and assocated public health surveillance and communication.

Recommendations

National authorities should formulate national systems including; policy, plans, national guidelines and tools to implement a recreational water safety framework through the following recommendations, summary guideline values and management advice for specific risks aligned with the recreational water safety framework. If not already established, clear roles and responsibilities among national and local authorities needs to be defined for each element of the framework.

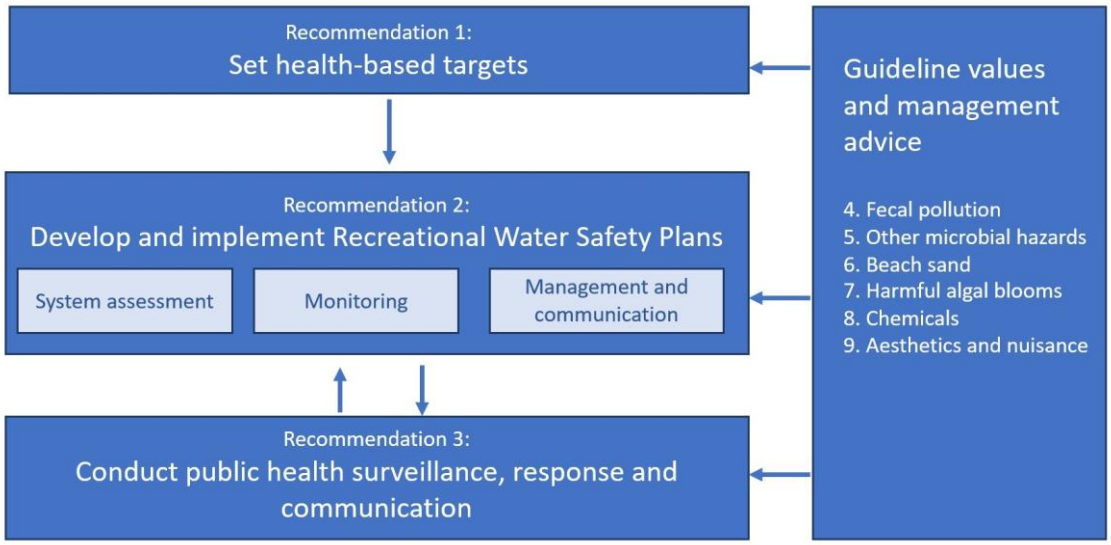


Fig. 0.1. Recreational water safety recommendation and management advice framework

*Chapter 9 includes some physical hazards

Recommendation 1: Establish national health based targets for recreational water bodies based on a national definition of tolerable risk and risk assessments

Sub-recommendations:

(…)

Recommendation 2: Develop and implement recreational water safety plans (RWSPs) for priority bathing sites

Sub-recommendations:

(…)

Recommendation 3: Conduct ongoing public health surveillance, response and communication of recreational water related illness

Sub-recommendations:

(…)

Management Advice 4: Fecal Pollution

(…)

Management Advice 5: Other Microbial Hazards

(…)

Management Advice 6: Beach Sand

Indicator:	☐ Intestinal enterococci in in both marine and freshwater (optional) *
Provisional Guideline value:	☐ 60 CFU/g # # Local epidemiological and quantitative microbial risk assessment studies are encouraged to establish guideline values for beach sand. In the absence of guideline values, efforts should focus on preventive measures described in management and communication below. * Preliminary evidence based on a pan-European average also suggests an indicative guideline value of 89 CFU/g for Fungi of wet weight.
System Assessment:	☐ Incorporate risk factors for pathogens of concern in beach sand within RWSP system assessment paying particular attention to beaches that are vulnerable from a physical and geomorphological perspective (enclosed beaches with minimal wave action)
Operational and verification monitoring:	• Pathogen monitoring follows sampling and analysis described in section 6.5.1 • Indirect monitoring (e.g dogs and birds on beaches)

Examples of management and communication:	For health authorities and water managers: • Limit access to the beach by dogs and feral animals, such as cats. • Prepare management plans for birds. • Provide properly designed solid waste disposal facilities. • Provide toilet facilities and appropriate wastewater and sludge treatment and stormwater drainage. • Conduct beach grooming to eliminate visible solid waste (taking care to minimize impacts on sand ecology). • Check source sand quality if beach sand re-nourishment is used to build artificial beaches or restore natural beaches. • Added strategies should be applied for beaches that are vulnerable from a physical and geomorphological perspective (enclosed beaches with minimal wave action). For recreational water and beach users (in the absence of environmental measurements). • Use a towel when sitting on the beach. • Wear shoes to minimize cuts when walking on the beach. • Protect open wounds from water and sand exposure. • Beach clean-up workers may be encouraged to wear protective clothing, including gloves and possibly dust masks. • Shower upon leaving the a beach. For public health authorities: • Stay in contact with lifeguards for potential on-site outbreak reporting. • Proactively intervene by contacting in medical centers – Remind staff to be alert to possible beach-related outbreaks and ailments.

Management Advice 7: Harmful algal blooms

(…)

Management Advice 8: Chemicals

(…)

Management Advice 9: Aesthetics and nuisance

(…)

1. Introduction

(…)

2. Recreational water safety planning

(…)

3. Public health surveillance, risk communication and engagement

(…)

4. Faecal pollution

(…)

5. Other microbial hazards

(…)

6. Beach sand

Beaches consist of the unconsolidated sediment that lies at the junction between water (oceans, lakes and rivers) and land; they are usually composed of sand, mud or pebbles. Sand beaches are sought after for recreation. In some cases, especially at higher latitudes, a significant proportion of time is spent on the beach rather than in the water. Activities involving sand may include beach-side sports and playing with sand, which have health benefits through exercise and recreation.

Microorganisms are a significant component of beach sand – bacteria, fungi, parasites and viruses have all been isolated from beach sand, and some are potential pathogens. Accordingly, concern has been expressed that beach sand or similar sediments may act as reservoirs or vectors of infection, as well as a source of water contamination (Whitman et al., 2014; Solo-Gabriele et al., 2016; Weiskerger et al., 2019).

This chapter describes microorganisms in beach sand, links to human health and recommended management actions.

Other hazards that affect beach sand quality include chemical contaminants (see Chapter 8); and the presence of solid wastes and plastics on the beach, insects, and sea wrack (see Chapter 9).

6.1. System assessment

Table 6.1 provides data on infectivity and concentrations observed in beach sand for selected microorganisms.

Table 6.1. Selected microorganisms in beach sand

Microorganism	Disease/role	Sources	Infectivity (lowmediumhigh)	Type of data available	References
Bacteria					
Campylobacter spp.	Gastroenteritis	Animal and human faeces	Low (800-10^6 CFU)	Prevalence data	Yamahara et al. (2012)
Clostridia	FIO	Animal and human faeces	NA		
Escherichia coli	FIO	Animal and human faeces	High (1-100 CFU)	Quantitative data	Abdelzaher et al. (2010) Shah et al. (2011)
Intestinal enterococci	FIO	Animal and human faeces	NA	Quantitative data	Abdelzaher et al. (2010) Shah et al. (2011)
Salmonella spp.	Gastroenteritis	Animal and human faeces	Low (>10^5 CFU)	Prevalence data	Viji et al. (2019)

Microorganism	Disease/role	Sources	Infectivity (lowmedium-high)	Type of data available	References
Staphylococcus aureus	Skin infections, sepsis	Humans	Low (>10^5 CFU)	Prevalence data	Thapaliya et al. (2017)
				Quantitative data	Abdelzaher et al. (2010)
				Quantitative data	Plano et al. (2013)
Pseudomonas aeruginosa	Ear, respiratory and skin infections; sepsis	Natural	NA	Quantitative data	Tugrul-Icemar & Topaloglu (2011)
Vibrio alginolyticus	Ear and wound infections, gastroenteritis	Natural	NA		
Vibrio parahaemolyticus	Ear and wound infections, gastroenteritis	Natural	Low ($10^7$$10^8$ CFU)		
Vibrio vulnificus	Ear and wound infections, gastroenteritis, sepsis	Natural	NA	Presence/absence data	Abdelzaher et al. (2010)
Vibrio cholerae non-O1/O139	Ear and wound infections, gastroenteritis	Natural	Low ($10^3$$10^8$ CFU)		
Viruses					
Bacteriophages	FIO	Animal and human faeces	NA		
Norovirus	Diarrhoea	Human faeces	High (~20 viral particles)	Presence/absence data	Abdelzaher et al. (2010)
Adenovirus	Diarrhoea, respiratory infections	Human faeces	Medium (~150 PFU)	Prevalence data	Monteiro et al. (2016)
Enterovirus	Gastroenteritis, fever, skin rash, conjunctivitis	Human faeces	High (<18 PFU)	Presence/absence data	Abdelzaher et al. (2010)

Microorganism	Disease/role	Sources	Infectivity (lowmedium-high)	Type of data available	References
Reovirus	Gastroenteritis, fever	Human faeces	NA		
Hepatitis A virus		Human faeces	NA	Presence/absence data Prevalence data	Abdelzaher et al. (2010) Monteiro et al. (2016)
Hepatitis E virus		Animal and human faeces	NA	Prevalence data	Monteiro et al. (2016)
JC Polyomavirus		Human urine	NA	Prevalence data	Monteiro et al. (2016)
Parasites					
Toxocara spp.	Diarrhoea, abdominal pain, malnutrition	Dog faeces	NA	Prevalence data	Bojar & Klapec (2018)
Ancylostoma spp.	Diarrhoea, abdominal pain, malnutrition	Cat or dog faeces Human faeces	Medium (~10 larvae)	Prevalence data	Bojar & Klapec (2018) Da Silva et al. (2009)
Tricuris spp.	Diarrhoea, abdominal pain, malnutrition	Human faeces	Medium (~10 larvae)	Prevalence data	Bojar & Klapec (2018)
Ascaris spp.	Diarrhoea, abdominal pain, malnutrition	Human faeces	Medium (~10 larvae)		Da Silva et al. (2009)
Giardia lamblia	Gastroenteritis	Animal and human faeces	Medium (10-100 cysts)	Presence/absence data	Abdelzaher et al. (2010)
				Quantitative data	Shah et al. (2011)
Cryptosporidium parvum	Gastroenteritis	Animal and human faeces	High (1-5 oocysts)	Presence/absence data	Abdelzaher et al. (2010)
				Quantitative data	Shah et al. (2011)
Fungi					

Microorganism	Disease/role	Sources	Infectivity (lowmedium-high)	Type of data available	References
Aspergillus spp.	Aspergilloma, aspergillosis, onychomycosis, allergy	l Natural	Opportunis t	Prevalence	Sabino et al. (2011)
Dermatophytes	Onychomycosis, tinea	Skin of animals and environmental (depending on species)	High	Prevalence	Sabino et al. (2011)
				16.7%	
Candida sp.	Candidosis (systemic and superficial infections)	Gut, skin and mucosae of animals	Opportunis t	Prevalence	Sabino et al. (2011) Sato et al. (2005)
Histoplasma capsulatum	Histoplasmosis (flu-like syndrome)	Guano of birds and bats	Medium	No data	NA
Blastomyces dermatitidis	Blastomycosis (flu-like syndrome)	Decaying vegetable matter	Low	No data	NA
Cryptococcus spp.	Cryptococcocal meningitis, pneumonia, systemic infection	Decaying vegetable matter and bird droppings (especially pigeons)	Low (medium for C. deuterog atii)	No data	Maziarz & Perfect (2016)
Cladophialophora bantiana	Cerebral infection (phaeohyphomy cosis)	Soil and rotten plant material	Low	No data	Sabino etal. (2014)
Fusarium spp.	Keratitis, onychomycosis, endophthalmiti s, skin infection, musculoskeletal infections	Water and plants	Opportunis t	Prevalence	Sabino et al. (2014)

FIO: faecal indicator organism; NA: not applicable

6.1.1. Faecal indicator organisms

Microorganism	Disease/role	Sources	Infectivity (lowmedium-high)	Type of data available	References

Faecal indicator organisms (FIOs; see section 4.2.3.1)) are nonpathogenic microorganisms that are used to indicate the degree of faecal contamination of the environment. FIOs include, Escherichia coli, intestinal enterococci, bacteriophages, Candida albicans and clostridia.

Thermotolerant coliforms and intestinal enterococci have been isolated from beach sand (Figueras et al., 1992; Signorile et al., 1992; Ghinsberg et al., 1994), and correlations have been found between contamination of beaches and contamination of adjacent seawaters (Oshiro and Fujioka, 1975, Aulicino et al., 1985; Roses Codinachs et al., 1988; Badilla-Aguilar and Mora-Alvarado, 2019).

Numbers of FIOs in recreational waters are correlated with the numbers of FIOs in adjacent beach sand (Phillips et al., 2011b). For recreational beaches, improved sand quality is often associated with improved water quality.

6.1.2. Bacteria

Staphylococcus aureus

The origin of Staphylococcus aureus (see section 6.2.2)) in beach sand is human activity. Its occurrence correlates with the number of swimmers on the beach (Papadakis et al., 1997; Plano et al., 2013). Many studies in the USA have demonstrated the presence of S. aureus in beach sand, including methicillinresistant S. aureus, at both marine beaches (e.g. Soge et al., 2009; Shah et al., 2011; Goodwin et al., 2012; Plano et al., 2013) and freshwater beaches (Thapaliya et al., 2017). A study conducted at 10 beaches in South Africa found that 100% of the S. aureus isolates evaluated showed multiple antibioticresistant patterns (resistant to three or more antibiotics) (Akanbi et al., 2017).

Pseudomonas aeruginosa and Vibrio spp.

P. aeruginosa (see section 5.2.1.3) has been isolated from beach sand in a number of countries (Mendes et al., 1993; Ghinsberg et al., 1995; Esiobu et al., 2004; Elmanama et al., 2005; Tugrul-Icemar & Topaloglu, 2011). Vibrio species (see section 5.2.1.5) have also been detected in beach sand (Elmanama et al., 2005; Abdelzaher et al., 2010; Shah et al., 2011; Viji et al., 2019).

Other Bacteria

Other bacteria that can be zoonotic, such as Campylobacter spp. and Salmonella spp., which mainly cause gastrointestinal infections in humans, have been isolated from wet and dry sand at beaches in a number of countries (Bolton et al., 1999; Vieira et al., 2001; Elmanama et al., 2005; Byappanahalli et al., 2009; Yamahara et al., 2012; Kahn et al., 2013; Shatti & Abdullah). Bird faeces may be an important source of these pathogens (Whitman et al., 2014).

6.1.3. Viruses

Relatively little work has been done on the presence in beach sand of enteric viruses that cause diarrhoea in humans. Viruses that have been detected in beach sand include enteric viruses, hepatitis A virus and human adenovirus (Nestor et al., 1984; Pianetti et al., 2004; Monteiro et al., 2016).

6.1.4. Protozoa

The zoonotic and human protozoan parasites Cryptosporidium spp. and Giardia spp. have both been detected in beach sand (Abdelzaher et al., 2010; Sato et al., 2005; Shah et al., 2011). These organisms cause gastrointestinal illness in humans.

6.1.1. Helminths

Beach sand has been found to contain eggs and/or larvae of the human and zoonotic parasites Toxocara spp. (roundworm), Ancylostoma spp. (hookworm) and Trichuris spp. (whipworm) (Schöttler, 1998; Da Silva et al., 2009; Bojar & Klapeć, 2018); Ascaris lumbricoides (roundworm) has also been detected (Da Silva et al.,

2009). Most helmints are transmitted via oral exposure, however, hookworms can penetrate the skin in contact with sand (e.g when walking or sitting on the beach).

Infections with these geohelminths are generally asymptomatic when patients are infected with a few worms; however, when infected with large numbers of worms, they may suffer from gastrointestinal disease (Ascaris, Trichuris, human Ancylostoma), and children's growth may be stunted (Ascaris, Trichuris). Toxocara larvae travel through the organs of infected people, causing fever, coughing, enlarged liver and pneumonia. Animal hookworms remain in the epidermis, causing cutaneous larva migrans presenting as pruritic rash (Heukelbach and Feldmeier, 2008).

Transmission of parasites to humans from beach settings has been documented during an outbreak of Ancylostoma spp. (feline hookworm) (Mann, 2010). The outbreak was linked to overpopulation of feral cats due to illicit feeding stations. Sporadic travel associated and endemic cases were reported from both tropical and temperate regions (Heukelbach and Feldmeier, 2008, Sow et al., 2017).

6.1.2. Fungi

Exposure to environmental fungi may lead to opportunistic infections, especially in immunocompromised people (Hoog et al., 2000). Superficial fungal infections are estimated to affect 20–25% of the world's population (Male, 1990); the responsible fungal species and prevalence vary by country and region (Havlickova et al., 2008). Some health problems favour the invasive process of serious fungal infections (Bongomin et al., 2017) – for example, asthma, cystic fibrosis, AIDS, cancer, organ transplantation and corticosteroid therapies. It is therefore desirable to limit exposure to fungi.

Dermatophytes (considered pathogenic and a dominant cause of superficial fungal infections) have been detected at beaches in Portugal (Sousa, 1990). Higher densities of beach users leads to higher levels of dermatophytes during the summer months (Brandão et al., 2002).

To date, relatively few studies outside Europe have looked at fungal contamination of beach sand. However, endemic fungal pathogens may be present in some regions, especially in inland water masses(Kidd et al., 2004; Antinori, 2014 Kantarcioglu et al., 2017;Miceli et al., 2019. Human migratory movements or expansion of habitats of fungi, e.g., due to climate change, are expected to occur with increasing frequency, thus promoting global spread (Datta et al., 2009; Weiskerger et al., 2019).

Candida albicans and other Candida spp. have been detected in sand beaches around the world. Emerging pathogens should be considered when addressing beach sand and possible deposition by nearing waters – for example, the multidrug-resistant and higher-salinity-tolerant Candida auris (JefferySmith et al., 2018). Some emerging species, and even some well-characterized and long-reported species, show increasing resistance to antimicrobials – for example, several species in the Aspergillus section Fumigati (Alcazar-Fuoli et al., 2008), a common beach sand contaminant that has reportedly caused infections in hospitalized patients in the Netherlands (Warris et al., 2003).

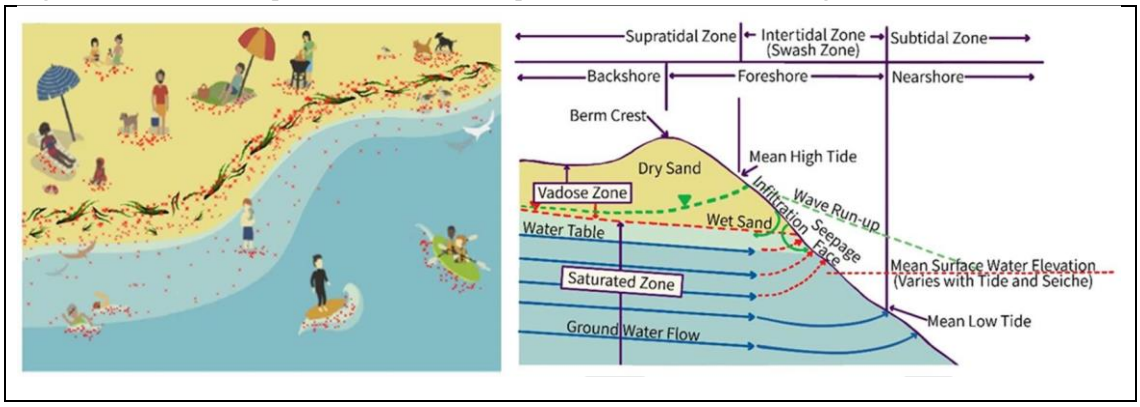
Information on infection resulting from fungal inhalation specifically from sand is unavailable. However, exposure to fungal spores can trigger an immune response (Buskirk et al., 2014; Tanaka et al., 2015). The public should be informed about the presence of allergenic fungi.

6.1.3. Cynaobacteriabacteria and cyanotoxins

(…)

6.2. Dispersion and fate of microorganisms in beach sand

Fig. 6.2 shows a conceptualization of the dispersion and fate of microorganisms in beach sand.



Note: The vertical aspect is intentionally exaggerated.

Fig. 6.2. Conceptualization of dispersion and fate of microorganisms in beach sand

Red spots in Fig. 6.2 represent the distribution of FIOs within the beach. The panel on the left emphasizes the distribution of various sources; the panel on the right emphasizes transport along the wave-impacted shoreline, including the freshwater definition of the foreshore and the marine water definition of the intertidal zone. The figure illustrates the seepage face for times when the mean surface water elevation is below the groundwater table (shown by the dotted lines). It shows infiltration that occurs when the surface water level rises above the groundwater table (shown by dashed lines), as typically occurs during wave run-up. The inverted triangles mark the lines that define the water table for each of these conditions.

6.2.1. Sources of microorganisms

Microorganisms are natural inhabitants of beach sands. Levels of pathogenic microorganisms in beach sands can increase through direct deposition from humans and animals (e.g. dogs, birds, wildlife). Microorganisms can also be introduced to sand from runoff and other sources introduced through water, such as from sewage, septic tank effluent and faecal sludge or bather shedding, which can be carried onto the sand by waves and tides (Whitman et al., 2014) but river-based beaches may have a dynamic of their own (Whitman et al. 2006). Atmospheric processes may also carry microorganisms from local faecal sources (e.g. farms, wastewater plants) and from the global circulation of dust (Kellog et al., 2003).

6.2.2. Proliferation of microorganisms

Once introduced, microorganisms can persist and potentially multiply in the beach environment in response to environmental factors, including availability of moisture, sunlight (Nelson et al., 2018) and nutrients. The availability of nutrients can be influenced by the presence of submerged vegetation and wrack along the shore (Weiskerger et al., 2019). Temperature influences survival of bacteria in sand: FIO concentrations can increase over temperature ranges from 4 to 44.5 °C (Alm et al., 2006; Byappanahalli et al., 2006).

The persistence and proliferation of microorganisms in beach sands may be facilitated by the formation of biofilms (Piggot et al., 2012), formed from bacterial secretions. Biofilms create microenvironments that can benefit microorganisms by providing access to nearby nutrients, and protection from harmful chemical and biological agents.

The environmental conditions conducive to survival and proliferation mean that background levels of microorganisms, including FIOs, may be higher in tropical and subtropical climates than in temperate regions (Fujioka et al., 1999, 2001) but this concept has been challenged by Byappanahalli et al. (2003)..

6.2.3. Influence of environmental factors

Various physical and geomorphological factors may encourage the survival and dispersion of FIOs and pathogens on beach sand. These include waves and tidal phenomena (see Fig. 6.2). Higher levels of sand microorganisms are observed at beaches with low-energy wave conditions (Gao et al., 2015; Feng et al., 2106). Thus, enclosed beaches generally accumulate more microorganisms in the sand than direct ocean-facing beaches. Waves lead to infiltration of large quantities of surface water and associated constituents (e.g. FIOs and nutrients across the beach face; Vogel et al., 2016). During periods of extreme wave conditions, such as hurricanes, the sediments are washed out and eroded, resulting in exposure of sand with lower microorganism levels (Roca et al., 2019). If the waves carry pollutants, the opposite may be observed immediately after hurricane conditions (Suzuki et al., 2018)) but there may be a delay in the migration of the contaminants in either direction due to cumulative effects.

Tidal fluctuations (or, in freshwater systems, water fluctuations due to lake standing waves) also drive water across the beach face. Infiltration captures FIOs in the upper intertidal region, and exfiltration leads to FIO loss at the lower tide mark (Gast et al., 2015; Wu et al., 2017). The area with the highest levels of FIOs on tidally influenced beaches is the sand just above the high tide mark (Abdelzaher et al., 2010; Whiley et al., 2019); for lakes, it is the backshore (Cloutier & McLellan, 2017; see Fig. 6.2 for locations). These areas may have ideal moisture conditions for prolonged persistence. As a result, the sand has been identified as the source of bacteria to the adjacent waters in many studies; levels of bacteria in water decrease with distance from shore (e.g. Tyner et al., 2018).

Urbanization in the vicinity of the beach and periods of heavy beachgoer use have been associated with higher microorganism levels (Aragonés et al., 2016; Villacampa et al., 2017; León-López et al., 2018).

Sediment type may also affect microorganism levels (Hernandez et al., 2014; Abreu et al., 2016; Villacampa et al., 2017). The presence of microplastics in sand has been associated with elevated pathogen levels (Curren & Leong, 2019).

6.3. Linking human health to beach sand quality

Methods to relate sand quality to human health include epidemiological studies and risk assessments.

6.3.1. Epidemiological studies

Evidence exists to link beach activities, beach sand quality and human health impacts. Box 6.1 describes an outbreak associated with sand (Brandão et al. 2020). Other epidemiological Epidemiological studies have linked sand contact with gastrointestinal illness (Bonilla et al., 2007; Lamparelli et al., 2015) and skin symptoms (Esiobu et al., 2013; Praveena et al., 2016).

Box 6.1 The case of an outbreak from sand in Azores, Porto Pim beach
Thirty people (mostly children) experienced an episode of skin rash days after a sand sifting beach operation at Porto Pim Beach in Faial, Azores during June 2019. An environmental and epidemiologic investigation was conducted to identify the cause of the outbreak of skin rash. The epidemiologic investigation found that some of the patients experiencing symptoms had never entered the beach water. During the pollution period and throughout the epidemiologic investigation, faecal indicator bacteria levels in water remained under the limits used for an Excellent designation for coastal bathing water. Thus sand contact was considered as a likely

primary exposure route. Sand microbiological analysis for faecal indicator organisms and electron microscopy strongly suggested faecal contamination. Chemical analysis of the sand also revealed a concomitant substance compatible with sodium-hypochlorite as analysed using gas chromatography and subsequently confirmed by free chlorine analysis. Inspection of the toilet facilities and sewage disposal system revealed a leaking sewage distribution box. Collectively, results suggest that the cause of the outbreak was the leaking underground sewage distribution box that serviced the beach toilet facilities, where sodium-hypochlorite was used for cleaning and disinfection. This sewage then contaminated the surficial sands to which beach goers were exposed. Chlorine being an irritant substance, was believed to have been the cause of the symptoms given the sudden presentation and dissipation of skin rashes. No gastro-intestinal illness was reported during this episode and during the following 30 days. Source: Brandão et al. 2020

6.3.2. Quantitative microbial risk assessment

Quantitative microbial risk assessment (QMRA) provides an alternative to epidemiological studies for assessing health risks from beach-associated pathogens (Haas et al., 1999; Ashbolt et al., 2010; Jang & Liang, 2018). QMRA methods are generally less expensive and less time-consuming than epidemiological studies; however, the relationships needed for calculating risks and disease rates are not always available (e.g. dose–response relationships for some microorganisms).

QMRA has been applied to estimate health risks from exposure to beach sand. Applying a set level of risk of gastrointestinal illness (19 cases per 1000 swimmers) to beach sand, Shibata (2012) calculated acceptable risks at <10 oocysts/g sand for Cryptosporidium, <5 MPN (most probable number)/g sand for enterovirus, and <106 CFU (colony forming units)/g sand for Staphylococcus aureus. Sabino et al. (2011) recommended maximum levels of 15 CFU/g for yeasts, 17 CFU/g for potential pathogenic fungi, 8 CFU/g for dermatophytes, 25 CFU/g for E. coli and 10 CFU/g for enterococci.

6.4. Monitoring

6.4.1. Sampling and analysis

Sand is a heterogeneous matrix, so sampling requires a collection of fractions (aliquots) to build a representative whole (composite), which should include problematic spots – that is, a worse-case scenario (Brandão, 2019). Sabino et al. (2011) analyzed composites of three supratidal equidistant grab samples combined and homogenized. This option may be mildly representative of an entire beach, compared to incremental sampling as described by Hadley et al. (2013). However, the history of monitoring a site will eventually define a normal pattern and identify outliers, no matter the sampling frequency or number of fractions used. Sites with no history might require more intense sampling, both in number of grab-samples and in frequency, until a pattern can be established.

Typically, sample analysis requires enumeration of the microorganisms in a specific mass of sand, on either a gross weight or dry weight basis. To report microorganism concentrations on a dry weight basis, a separate aliquot of the sand is analysed for moisture content. The most common method to enumerate microorganisms in sand is through extraction.

Historical analytical results may establish an initial water quality assessment of microorganism concentrations that will help detect sporadic pollution events (Brandão, 2019).

Box 6.1 describes recommended sampling and extraction procedures.

Box 6.1. Beach sand sampling and analysis

Sampling of beach sand

- Select the sand area of the beach mostly used (usually the supratidal area of the foreshore of the beach - see image 6.2 for definitions)
- Use sterile sampling spoons to collect several shallow aliquots from the surface in the target area (up to 10 cm deep). If more control over sand depth is necessary, shallow cores can also be used instead of scoops to ensure a uniform sampling depth.
- Place the aliquots in a sterile container.
- Thoroughly mix the aliquot before selecting a subsample for analysis.
- Use of standardized methods for sample collection is encouraged (e.g. parts 9, 12, 15 and 19 of ISO 5667: Water quality – sampling).

Extraction of microorganisms from beach sand

FIO (Boehm et al., 2009)

- Use a 10:1 ratio of eluent volume (usually 100 mL) to sand weight; the eluent is phosphatebuffered saline or deionized water.
- Shake by hand for 2 minutes.
- Allow to settle for 30 seconds.
- Analyse the eluent in a similar way to water.

Fungi

- Use gentle orbital shaking (Sabino et al., 2014) in extraction fluids such as water or saline solutions; extraction cannot be violent because of the risk of hypha breakage (generating extra colony forming units).
- Use of Tween may aid extraction of less hydrophilic species, such as Penicillium and dermatophytes.

6.4.2. Guideline values

The recommended provisional guideline value for beach sand is 60 CFU/g of intestinal enterococci based on the derivation below.

Assessing the relative risk of exposure to sand versus water requires setting an equivalency between the uptake of microorganisms from water versus uptake from sand. The equivalency would correspond to the 200 intestinal enterococci CFU/100 mL for water ingestion. Values of water and sediment consumed are available in the literature. Seawater ingestion rates by children during swimming have been estimated at 30 mL (Schets et al., 2011). Estimated sand ingestion rates for children are variable, depending on whether the children have pica tendencies (i.e. an above-normal tendency to consume soil). The low end of soil consumption for a child with pica tendencies is estimated at 1000 mg/day (USEPA, 2011). For children without pica tendencies, the consumption rate is estimated at 190 mg/day (Van Wijnen et al., 1990). The equivalent enterococci

concentrations in sediments would correspond to 60 CFU/g assuming pica child sand consumption rates. With assumptions about ingestion rates of seawater and sand, a very rough estimate of acceptable levels of enterococci in sand, C_s (units of CFU per mass of sand), can be established as per following equation:

$$C_s = \frac{C_w V_w}{M_s}$$

Where Cw is the concentration in the water, Vw is the volume of water consumed, and Ms is the mass of sediment consumed per beach visit. However, the above expression is dependent upon a significant assumption: that the ratios and uptake of enterococci and pathogens is the same for both water and sand. The 60 CFU/g (wet weight) is within the same order of magnitude as the 10 CFU/g level recommended by Sabino et al. (2011) (see section 6.4.2). Assuming equivalent pathogen ratios and uptake rates, these values can be used provisionally as a rule of thumb to determine whether beach sand is in need of improved management to reduce FIOs.

Although no set guideline values can be provided for other microorganisms in beach sand, local epidemiological and QMRA studies are encouraged to establish such values (risk-based and local characterization approaches). Recently, a pan-European initiative has established 89 cfu/g of sand as site-blind average value for fungi (Brandão et al. 2021). Further work on Fungi and other biological groups are necessary to establish actual exposure thresholds to use in analytical recommendations. In the absence of guideline values, efforts should focus on preventive measures. Management, education and communication (see section 6.6) are important precautionary measures, as are components of local water safety plans (see Chapter 2).

6.5. Management and communication

A sanitary survey is a first step in evaluating pollution sources for beach sand (see Chapter 2). This involves documenting potential sources of faecal contamination of sand.

6.5.1. Management actions

Animal excreta – including that of dogs, birds and other locally significant animals – increases FIO levels and introduces pathogens in beach sands. Exercising of dogs should be avoided in beach areas. The exercising of dogs should be kept separate from human use during bathing seasons. Sections of the coast should be designated for this particular purpose. Access to the beach should also be limited for feral animals, such as cats, using humane and culturally sensitive methods. Management plans should be put in place for managing birds, whether native (protective measures) or non-native (deterrent measures). Increased public awareness may help to reduce exposure to feral animals and birds, and minimize feeding of these animals. Beach cleaning may remove some animal excreta, but it is more often undertaken for aesthetic reasons, or to remove litter or sharp materials, such as broken glass.

Other management strategies for beaches include proper design of solid waste disposal facilities, provision of toilet facilities and appropriate stormwater drainage (Kelly et al., 2018).

- Garbage disposal should be available in designated areas; the garbage should be covered to minimize access by animals and should be protected from rainfall.
- Proper solid waste management will help to minimize the presence of non-native bird species that can contribute FIOs to the beach sand environment.

- The availability of toilet facilities at the beach can minimize FIO impacts from humans who visit the beach, and will also encourage proper hygiene practices, such as more frequent handwashing, during beach visits.
- Drainage systems should be appropriately designed at beach areas; drainage from parking lots and nearby areas should not be permitted to flow directly onto the beach.
- Direct stormwater drainage from surrounding communities onto the beach should be discouraged. If outdated infrastructure allows drainage of stormwater onto the beach, access to waters downstream should be restricted to avoid contact by beachgoers.

In some countries, particularly at resort areas, mechanical sand cleaning or beach grooming is used to eliminate visible solid waste mixed with sand. This reduces the amount of organic matter such as seaweed and therefore reduces development of microorganisms. Care should be taken in choosing the beach grooming strategy to minimize impacts on the sand quality (Kinzelman et al. 2004) and ecology (Llewellyn & Shackley, 1996).

Disinfection of sand (e.g. with chlorine, iodine, ultraviolet irradiation or thermal treatment) is not recommended because of negative impacts on native flora and fauna. Alternative simpler methods, such as sifting and aeration, could be applied (Figueras et al., 1992), together with beach supervision to minimize inputs and sources.

Beach sand renourishment is practised at some sites to build artificial beaches and restore natural beaches subjected to erosion. This consists of fortifying a beach with sand translocated from an external site – offshore sources, sand quarries or another beach. The source of the sand and its quality should be considered in developing a beach renourishment plan, to preserve native ecosystems and avoid importing non-endemic arthropods. Quality considerations for the imported sand should include its microbiological and chemical quality, and mineralogy.

Human faeces is the major risk factor in areas without safe sanitation services. Sewage dumping should not be practiced in the proximity of recreational areas.

6.5.2. Communication

Education and communication campaigns can include signage about policies concerning dogs, feeding of wildlife and disposal of trash. The location of toilet facilities should be identified. Beachgoers should be encouraged to practise good hygiene, such as using clean towels while on the beach, washing their hands before eating and showering immediately after beach visits. They should be encouraged to wear shoes to minimize cuts when walking during beach visits. Use of the beach should be discouraged if an individual has significant wounds; minor wounds can be covered with waterproof bandages.

More details about dissemination of educational materials are provided in Chapter 3, but Box 6.2 provides some suggestions.

Box 6.2. Suggested communication messages for the general public and beach managers

Communication for the general public

- When visiting the beach, leave nothing behind but your footprints. You may even help clean up if you see an item of solid waste.
- Shower thoroughly when you get home, but also use the showers at the beach. Make sure you wash off sand from your skin and from the inside of your ears.
- If you have wounds, dress them properly with waterproof bandage before you go to the beach and avoid exposure to water; otherwise the wound may get infected.
- Don't rub your eyes if you have sand in them; rinse with clean water instead. Rubbing may cause abrasions that might result in infections.
- Do not take pets to the beach. Take them to nonbathing areas instead.

Communication for beach managers

- Conduct sanitary inspections to identify possible sources of contaminants and develop a plan to manage these sources.
- Keep litter contained, and make sure it is removed at the end of the day, to avoid foraging by feral animals during the night.
- Develop a management plan for controlling birds and feral animals.
- Develop a policy concerning dogs, and enforce the policy.
- If ecologically acceptable, develop an appropriate sand grooming plan.
- Provide signage for beachgoers to encourage appropriate beach use, and inform them about possible health risks.

6.6. Research needs

Studies are needed to establish beach guideline values for acceptable levels of microorganisms in beach sands. Epidemiological studies that include sand measures, detailed documentation of child play activities, and follow-up concerning possible health outcomes would be ideal to establish the relationships needed to confirm acceptable levels of FIOs for beach sands; particularly, for emerging concerns like opportunistic Fungi, which current water quality recommendations do not contemplate. More information about non-point sources including birds, macro-algae, forest and agricultural runoff as well as storm runoff is desired and the ability of sand to convey contaminated groundwater remains obscure and unsettled

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A Murine Inhalation Model to Characterize Pulmonary Exposure to Dry Aspergillus fumigatus Conidia

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In silico modeling of spore inhalation reveals fungal persistence following low dose exposure

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7. Harmful algal blooms

(…)

8. Chemicals

(…)

9. Aesthetics and nuisance

(…)

Glossary

(…)

Index

(…)

Annex 1: (microbial methods TBC)

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Chapter 9

Discussion

9.1 General discussion

Understanding microbial communities in sand, their inter-species interactions, level of self-protection, both physically, as they enclose themselves in multi-communal biofilms, and chemically, by using molecular warfare against other susceptible species like antibiotics and statins (Macreadie *et al.* 2006; Cornforth and Coster, 2015; Weiskerger *et al.* 2019), is a fundamental requirement to be able to predict and mitigate surges at the beach. Brandão *et al.* (2020) – Chapter 7, and Heaney *et al.* (2012) demonstrate in real life that sand contaminants do matter and that its quality needs to be monitored just as much as water does, although it has been very much off the radar of any regulation until very recently. In 2017, Argentina included sand inspection for rubbish in its water quality standards as an exclusion parameter (Ambiental, D.d.s., 2017) and Lithuania added monitorisation of helminths in sand in 2018 to their 2007 National regulation (Ministry of Health of the Republic of Lithuania, 2018).

The new recreational water guidelines (WHO, *in press*) advise sand monitoring with reference values for enterococci as Faecal Indicator Bacteria (FIB) and for fungi. But are those enough to ensure the safety of beach visitors of all ages and clinical singularities, e.g., cystic fibrosis, allergy to a particular fungus or even transitory immune impairment, that expose themselves to direct contact and sometimes even complete immersion for fun? Probably not. Ultimately, regulation can only go so far. If the community feels that it needs to go beyond a regulated minimum, then that community may use additional parameters. Analytical methods, however, are issued by default by the International Standards Organisation, upon adoption of voted out of a pool of possible candidate methods, and after an air-tight screening of all possible go-wrong scenarios. The European Commission for example, issues water quality Directives listing a reference method and proven equivalent methods. Each individual member-state may however opt for yet another unlisted method, provided that it passes a 3 experts panel that contemplates reproducibility, equivalence with reference method and representativity, both geographical and analytical for different concentrations, all supported by a statistical analysis. Upon acceptance, the study needs to be replicated in other Member-States that wish to integrate that non-reference method in their own list of options, due to geographical discordance.

Another aspect not covered by the WHO, yet that also falling in the category of methods or procedures, is the sampling itself; the frequency and manner in which samples are collected and transported to a laboratory for processing. Brandão (2019) - chapter 3 - addresses this subject and so does Brandão *et al.* (2021a) - chapter 8, to some extent. Now that the new WHO guidelines (WHO, *in press*) are soon to be published with a sand monitoring recommendation,

the philosophy behind the sampling really needs to be defined soon. This will render monitoring efficient and equivalent between different communities – no doubt, for comparability of results obtained and stories learned in different places.

Different regions of the globe will always present specific features in public health protection from exposure to microbial life - specific regions implies addressing endemic pathogens. In the tropics, e.g., the existence of percutaneous infection by nematodes like hookworm and schistosomiasis can be a serious problem for beach users (Krzywanski *et al.* 2012; Weatherhead, 2017).

Climate change may also alter the current paradigm of zoonoses and many endemic infections. Understanding how climate changes may influence the sand and water microbiota was one of the objectives of this thesis ("study the effects of a changing Earth on predicting microbial dynamics and human health risks in the beach water/sand continuum"). Weiskerger *et al.* (2019) - chapter 2, aimed at envisioning future scenarios of waterborne and sand transmitted diseases in a future replenished of climatic alterations and extreme weather events, such as hurricanes, droughts, heat and cold waves and desertification, extreme precipitation, and strong windstorms. On that note, Fujioka *et al.* (2015) and Teixeira *et al.* (2020) addressed the very same subject with one common target: quality indicators that in the future will need to be adjusted in many regions, to reflect emerging pathogens; endemic and otherwise. Fungi have not been left out of these publications but one emergent in particular is the cause for great concern, due to its unknown ecological niche and widespread field of action. *Candida auris* has spread now to all continents and has been detected only in hospitals and colonising those that were hospitalised. This species is mimicking the successful path of Methicillin-resistant *Staphylococcus aureus* (MRSA) which slowly entering the community (Chalmers and Wylam, 2020). MRSA was reported in 2009 as present in beach sand and water in California, USA, by (Goodwin and Pobuda, 2009). Considering that colonisations of *C. auris* beyond the hospital are a fact, it should be only a matter of time before it can also be found in beach sand and water around the world; especially given its biofilm producing nature, which grants protection from the elements to the cells lying in more central sections of the biofilm, as reported by Uppuluri *et al.* (2020).

Biofilms were addressed in detail in Weiskerger *et al*. (2019) - chapter 2. Their presence makes the pollution mitigation challenging. The biocides, if need use, do not go beyond the superficial layers, unless a dispersing agent is added. Conversely, in the laboratory, the biofilms may hold

back some of the colony forming units, but those would also not readily available as inoculums to form new colonies in the natural environment. Brandão *et al.* (2020) - chapter 7, presents pictures of initial stadia of biofilm construction on the surface of sands of grain, revealed in electron microscopy (Fig. 3 A and B of the publication). Biofilms may be complex, multi-species communities that integrate fungi. When black mould is a constituent of a biofilm exposed to sun radiation, its presence will protect other species from the damaging effects of ultra-violet radiation by absorbing it as a source of energy for its own growth. As carbon source it will use either decaying bio-film inhabitants or simply us hydrocarbons present by fossil-fuel residue or shedding by more complex lifeforms visiting or inhabiting the beach (Babič *et al*. 2018). Some of the dematiaceous fungi are toxinogenic. In fact, many fungi are either toxinogenic or allergenic. Aleksic *et al.* (2017) describes the difference between the propagation of aerolised mycotoxins (mycophenolic acid, sterigmatocystin, and macrocyclic trichothecenes) of three common fungal indoor contaminants originating in the environment: *Penicillium brevicompactum*, *Aspergillus versicolor*, and *Stachybotrys chartarum*. Considering the outdoor dispersion of any biochemical molecules, the toxinogenic traits of fungi should were not heavily explored for this thesis. Regarding the allergenic potential of fungal exposure, Buskirk *et al.* (2014) showed in a murine model that the dry exposure twice a week to 10^5 spores of *A. fumigatus* triggers an inflammatory response in the lungs at 24 and 48 hours thereafter and an IgG elevation after 7 days, concomitant with spore germination. Tanaka *et al* (2015) determined that cytokines release in immunocompetent individuals takes place about 19h post-exposure and Green *et al*. (2006) determined that fungal fragments take the lead over spores in lung surface deposition. These data suggest a delayed first response to the exposure to fungal allergens but also a necessity to inform the public of presence of allergenic fungi.

Fungi may also be indicators of pollution, as indicated in 1.6.1, in Routine. In Brandão *et al*. 2020 – chapter 7, the fungal analysis was partially compatible with the faecal pollution, and partially compatible with vegetable debris. Upon on-site description of the problem that originated the study, the local team described how a tropical winter storm delivered enormous amounts of ocean and near coast debris into the cove, including high quantities of decaying vegetable matter. That too was reflected in the fungal analysis, by the considerable presence of *Fusarium* spp, of *Aspergillus* section *Circumdati,* and some *Candida tropicalis* which are common plant pathogens and colonisers. Black mould consumes carbohydrates, organic and

inorganic of origin, and were therefore non-informative of either the storm or the faecal contamination. The same applies to *Rhodotorula* spp.

Brandão *et al.* 2002 showed that the human element pressure on a beach is clearly visible on the level of fungal contamination of the sand. That studied selected three different kinds of beaches of several regions of Portugal. Sampling took place every two months for 13 months. The three types were wild beaches, beaches with water quality problems and beaches awarded blue flag. Blue flag beaches are high maintenance beaches compared to the other two types and yet they were not the best ones in terms of fungal quality of the sand. Instead, the beaches with lesser use showed the best results. Fungi are thus very revealing of hygienic aspects of beaches and sources of nearby contamination. Sporulating fungi can travel but yeasts and dermatophytes do not, unless strong winds raise sand carrying these types of fungi. They are otherwise always the result of direct deposition or shedding.

Considering all the above, it should come as no surprise that the WHO chose a total fungal count value of Brandão *et al.* 2021b – chapter 6 to be indicative for beach managing purposes rather than a parameter value. This chapter is the answer to a main objective of this thesis ("Run a collaborative study on fungal diversity in the sand and water of recreational water bodies of Europe and Sydney, Australia"). The historical data of a beach will always reveal the extraordinary results associated with any influencing event (e.g., windstorms, heavy rain events and beach festival parties), as described in Brandão 2019 – chapter 3 and Brandão *et al*. 2020 – chapter 4; which was another objective of this thesis ("Identify information on sand exposure that was missing in the scientific literature and fill in possible voids by reviewing literature and writing about it"). A future health-oriented set of safety parameters for fungi depends on epidemiological studies or Quantitative Microbial Risk Assessment (QMRA) estimations.

Weiskerger *et al*. 2020 – chapter 5 explains how to approach QMRA for fungi and points out how much information we are currently lacking, in terms of infectibility indexes of fungal pathogens and opportunists. All other variables of the assessments are known for bacteria, as described in Brandão et al. (2021a) – chapter 8, in section 6.4.2. Guidelines values.

Taking into consideration the data obtained in the present study, along with what has previously been published on this matter, the following sections fulfil the ultimate objective of this thesis by proposing a consensus in methods and parameters for: sand monitoring and analytical approaches, remediation, public information and also present a way forward on the theme sand quality for health protection of beach users.

9.2 Monitoring program

Sand is patchy and therefore monitoring is a challenge. In order to implement monitoring programs, several issues need to be addressed. Regarding sampling frequency, as explained above, the more points collected and analysed the more robust the information will be. But a cost efficacy balance needs to be found between the costs implicated in this course of action and the gains obtained in public health protection, many of which remain to be quantified. A general rule of thumb is that the official bathing season is the period that attracts most people to the beach and when maintenance takes place. Sampling outside this period serves only academic purposes and may eventually help beach management plan actions to improve results of sampling during the bathing season, e.g., cleaning the beach, planning the number of trash-bins, beach nourishment to increase dispersion of sunbathers and remove animal excrements. Sampling sand and water together seems like the most reasonable plan since sampling less often than water, may result in the lack of relevant comparison information between both matrices. In addition, worst-case scenarios are rather relevant in sand monitoring (known pollution spots due to run-off or around beach infrastructures, where beach visitors tend to congregate. Also, at the laboratory level, it is easier to process both sand and water at the same time, to manage results' reports which may be include both matrices, allowing a full view of simultaneous snapshots to be able to inter-relate and assess any directionality of discrepant pollution in sand and in water together. Regulation should thus recommend sampling together and leave the decision of sampling outside the bathing season to the beach managers.

Samples must always be transported speedily and cooled to the laboratory for processing within 24 hours, as recommended by Brandao *et al*. 2002, to avoid alterations of the microbiota. If cooled transportation is not available, differential tolerance to dehydration and transportation conditions may also alter the microbiota before the sample is processed at the laboratory. For this reason, also, upon arrival, samples must remain cooled until processing.

9.3 Analytical recommendations

The following points will provide expert recommendations on how to perform sand analysis. Some authors describe using crude sand as a sample and others choose to express the results per dry weight of sand, by determining the water content and discounting it from the sample preparation calculations. For Bacteria and Fungi, Sabino *et al*. 2011 describe a water extraction of 40 ml:40 g of sand for fungi and 500 ml:50 g of water: sand for FIB, followed by the respective procedures mentioned in some of the next points:

9.3.1 Faecal Indicator Bacteria

Both the European Bathing Water Directive (EU, EEU, 2006) and the United States Recreational Water Quality Criteria (USEPA, 2012) refer to the use of enterococci and *E. coli*. as FIB parameters for recreational/bathing waters classification by enumeration of Colony Forming Units (CFU) per 100 ml of volume of water. The USEPA version includes a molecular quantification also, as a faster alternative to allow closing a beach to public use in a shorter period of time. The enumeration is described in both regulatory documents as either the filtration method or the most probable number determination, in which a series of miniaturised tests allows an estimation of the most probable number of CFU/100 ml, as described by the international standards for enterococci ISO 7899-1 (International Standard Organisation, 2000a) and ISO 7899-2, (International Standard Organisation, 2000b), and for *E. coli*, ISO 7899-1 (International Standard Organisation, 2014), and ISO 9308-3, (International Standard Organisation, 1998). The classification is based on a geometric mean system, as explained in both regulatory documents.

The parameter enterococci has been associated with ailments in bathers by Kay *et al.* (1994). Based on these results that are still valid today, Brandão *et al.* (2021a) – chapter 8 sets a threshold in sand by QMRA estimation of the equivalent in sand for the threshold of water: "*Assessing the relative risk of exposure to sand versus water requires setting an equivalency between the uptake of microorganisms from water versus uptake from sand. The equivalency would correspond to the 200 intestinal enterococci CFU/100 mL for water ingestion. Values of water and sediment consumed are available in the literature. Seawater ingestion rates by children during swimming have been estimated at 30 mL (Schets et al., 2011). Estimated sand ingestion rates for children are variable, depending on whether the children have pica tendencies (i.e. an above-normal tendency to consume soil). The low end of soil consumption for a child with pica tendencies is estimated at 1000 mg/day (USEPA, 2011). For children without pica tendencies, the consumption rate is estimated at 190 mg/day (Van Wijnen et al., 1990). The equivalent enterococci concentrations in sediments would correspond to 60 CFU/g assuming pica child sand consumption rates. With assumptions about ingestion rates of seawater and sand, a very rough estimate of acceptable levels of enterococci in sand, C_s (units of CFU per mass of sand), can be established as per following equation:*

$$C_s = \frac{C_w V_w}{M_S}$$

Where Cw is the concentration in the water, Vw is the volume of water consumed, and Ms is the mass of sediment consumed per beach visit. However, the above expression is dependent upon a significant assumption: that the ratios and uptake of enterococci and pathogens is the same for both water and sand."

Boehm *et al*., 2009, describe several methods of extraction of FIB from sand for filtration analysis, using handshake and several sand elution buffers recommending a handshaking for 2 minutes extraction with 1:10 ratio of sand to distilled water or PBS, followed by analysis of 10 ml of the eluent (equivalent to 1g of sand), according to the ISO 7899-2 (International Standard Organisation, 2000b). Sabino *et al*., 2011, describe an extraction of 50g of sand with 500 ml of distilled water, followed by the use of Quanti-Tray® systems from IDEXX™ (IDEXX, Westbrook, MN, USA) to determine enterococci, coliforms and *E. coli* Most Probable Number (MPN) Colilert® and Enterolert®. This option was never compared with those described in Boehm *et al.* (2009) but does perform well in an inter-laboratory assessment scheme currently in place by the *Programa Nacional de Avaliação Externa da Qualidade* of the National Institute of Health Doutor Ricardo Jorge for several years. Other solutions, as long as relatively equivalent to the ones standardly used for water, like molecular amplification of nucleic acids and anti-body-based quantification are alternatives that may also be considered for faster and/or cheaper results, since there currently no standard methods for FIB analysis in sand.

9.3.2 Other Bacteria

Section 1.6.3 – Bacteria introduces two bacteria that may be of interest in sand analysis: *Pseudomonas aeruginosa* and MRSA. *P. aeruginosa* may easily be tested using an IDEXX™ (IDEXX, Westbrook, MN, USA) Quanti-Tray, just like FIB. The MPN for this bacterium is called Pseudolert®. MRSA analysis from sand has been described by Goodwin and Pobuda (2009), based on filtration of an eluent of sand 2g to 80 ml of PBS extraction, followed by vacuum filtration in .45 μm pore nylon membrane and incubated either on SCA or C-MRSA media (BD Biosciences, San Jose, CA, USA).

Focusing on non-FIB bacteria generally is recommended only is there is a clinical reason to look for any in sand, such as investigating outbreaks or monitoring endemic species.

9.3.3 Fungi

Sabino *et al.* (2011) describe the following analytical procedure: forty grams of crude sand (not dry weight) are extracted with 40 ml of sterile distilled water by orbital shaking for 30 min at 100 rpm and the extract is then plated (0.2 ml) in triplicates per media (Malt agar/Potato

dextrose agar with chloramphenicol and Mycosel agar (Cycloheximide, Chloramphenicol agars)). Plates are incubated for 5 days in Malt agar and 21 days in Mycosel agar, both at 27.5 °C± 0.5 °C. Identical colonies are counted and identified, if required to match the different study parameters and the results are given in colony-forming units (CFU) per gram of crude sand (equivalent), as mean numbers of each triplicate (0.2 ml X 5=1 ml or 1 g).

Echevarria (2019) describes an alternative method consisting of spread-plating 1g of sand directly on the culture medium, but that option was abandoned in the National Institute of Health Doutor Ricardo Jorge before Brandão *et al.* (2002), because plates often had mites migrating on the culture medium, leaving a trail of *de novo* fungal colonies behind. Those trails made reading individual colonies after incubation rather difficult.

Fungi in water were addressed in Brandão *et al.* (2021b) – chapter 6, but the conclusion of the study is that a lot more data needs to be collected before a recommendation can be issues. The same team is preparing to address in the future.

9.3.4 Helminths

Lithuania included helminths monitorisation in sand in 2018 and it is currently the only known regulation on Helminths, as mentioned in WHO (*in press*). The diploma states with the following instructions:

"17.4 The top layer of beach sand to a depth of at least 0.1 m must be cleaned and loosened (raked) (mechanically or manually) at least once a week. When pathogenic helminths or their eggs are found in beach sand, the authority responsible for the administration of the beaches and bathing areas must be informed and the top layer of sand must be loosened (raked) to a depth of at least 0.10 m as soon as possible, regardless of the work schedule.

Beach sand for human pathogens helminths and their eggs must be tested once before the start of the bathing season and monthly during the bathing season, i. y. at least four times:

1.1. sand samples must be selected by specialists from the laboratory examining the samples;

1.2. the first sand sample must be taken and analyzed two weeks before the start of the bathing season;

1.3. at least two sand samples from different beach locations shall be taken simultaneously from the beach for parasitological examination;

1.4. if there are playgrounds or sports grounds on the beach, sandboxes for children, additional sand samples must be taken from them. They must be sampled at least four times during the bathing season.

2. Beach sand sampling procedure:

2.1. Beach sand for parasitological examination (1 sample) is taken from an area of 25 m2;

2.2. taken from 5 to 10 points of 10 to 20 g from a depth of 2 to 3 cm. The total amount of sand sample must be between 150 and 200 g;

2.3. when taking a sample from a playground or sports ground, a sandbox for children, the sand shall be taken in accordance with the procedure set out in Sub-paragraph 2.2 of this Annex;

2.4. beach sand is sampled with a scoop or spoon into durable packages, bags or sealed containers. Stones, leaves or debris should not enter the samples.

3. Each package of sand samples shall be labeled with the place, date and authority responsible for the administration of the beaches and bathing areas. After completing the sampling cover letter, which contains the details of the authority responsible for the administration of the beaches and bathing areas, the place of sampling, the date, the purpose of the test and the contact details of the sampler, the samples are delivered to the laboratory for testing."

Ideally, this regulation should be tested an implemented in other countries as part of beach sand monitoring activities with relevance for human health protection.

9.3.5 Viruses

There is currently no regulation on viruses but instead they are considered to be indicated by FIB. However, attempts have been made to quantify viral genomes in order to determine their presence in sand. Hepatitis A and Adenovirus were detected in less than 10% of a pool of samples by Monteiro *et al.* (2016), followed direct extraction with commercial nucleic acids extraction kits. Viability tests did not produce any positive results. SARS-CoV-2 in sand is in study in Italy but the results are unpublished thus far.

9.3.6 Insects

There is currently no methodology published for detecting insects in beach sand. However, Brandão et al (2021a) – chapter 8 describe the species considered of interest by the authors: Mosquitoes, Biting midges, Sandflies, Flies and Blackflies.

9.3.7 Microbial Source Tracking

Microbial Source tracking may be required to determine the biological origin of faecal pollution, as described in 1.6.3 – Bacteria. The recommended method, currently being tested for sand use in the National Institute of Health Doutor Ricardo Jorge is Bacteroides based.

9.4 Sand remediation and disaster mitigation

The first approach to maintain a good level of microbial life in sand is to prevent contamination in the first place by implementing measures as described in 9.2 – Monitoring program. However, that may not always be possible. Brandão *et al.* (2019) and Brandão *et al.* (2021a) discuss possible actions to take for remediation in case of necessity. In this case, when the quality is documented as poor and action needs to be taken, chlorination is the approach most commonly used. Alternatively, sand may be replaced as a remediation approach, as described in Brandão *et al*. (2019).

Beach nourishment to mitigate loss of coast after a disaster should consider only nearby sand to use, as to avoid the introduction of foreign life from where the nourishing sand originates, as explained in 1.2 - Types of sand and artificial beaches.

9.5 Beach users and Public Health management

Educating the public and the beach personnel on how to maintain a healthy level of potential pathogens and how to act at the beach, and upon leaving the beach, is a crucial element in maintaining the good health of the beach professionals and the general public. WHO (*in press*) describes a certain number of actions in this perspective: *"Other management strategies for beaches include proper design of solid waste disposal facilities, provision of toilet facilities and appropriate stormwater drainage (Kelly et al., 2018).*

- *Garbage disposal should be available in designated areas; the garbage should be covered to minimize access by animals and should be protected from rainfall.*

- *Proper solid waste management will help to minimize the presence of non-native bird species that can contribute FIOs to the beach sand environment.*

- *The availability of toilet facilities at the beach can minimize FIO impacts from humans who visit the beach, and will also encourage proper hygiene practices, such as more frequent hand-washing, during beach visits.*

- *Drainage systems should be appropriately designed at beach areas; drainage from parking lots and nearby areas should not be permitted to flow directly onto the beach.*

- *Direct stormwater drainage from surrounding communities onto the beach should be discouraged. If outdated infrastructure allows drainage of stormwater onto the beach, access to waters downstream should be restricted to avoid contact by beachgoers."*

These measures alone may not maintain sand microbiota in acceptable levels all the time, but they result from years of many studies on diffuse pollution and hygiene concepts targeting health human protection in recreational water environments.

9.6 Way forward

Besides research to fill the many gaps currently existing and discussed throughout this dissertation, building a procedure to classify beaches according to their results of sand monitoring, as currently happens with water, is a necessity. Considering the non-normal distribution of fungal counts along time, using standard deviations and geometric means is not advised. A good alternative is to classify a beach as compliant or not compliant, allowing a certain number of results to fail, in case of ordinary microbiota fluctuation: 20% of rejection rate, for example, which is not unreasonable, in case of the fungi, considering the results of Brandão *et al*. (2021b) – chapter 6. This would mean a guidance value of 89 CFU/g of total fungi in sand but a rejection limit of the 80% percentile, which is 490 CFU/g. This means that during a sampling period, no more than 20% of the samples are acceptable to have values above 490 CFU/g.

For enterococci, the value stated in WHO (*in press*) theoretically reflects the same health effect of water thresholds. Therefore, care should be taken in samples exceeding the value of 60 CFU/g of sand. This value is considered provisional as it is the result of a QMRA calculation which does not contemplate the native flora of a beach. Epidemiological studies should be run in order to confirm the validity of assumptions of the calculation and also in large freshwater basins, FIB may not reflect actual faecal contamination but that can be further assessed by studying the microbiota at a genetic level, including running MST testing.

In the future, epidemiological studies combined with sand analysis of all locally relevant parameters should take place. The values used for the new WHO guidelines are at this stage provisional only due the lack of clinical expression back up. One option would be to run a self-assessment study of a large population visiting defined beaches like the one published by Leonard *et al*. (2018). Studying exposure in beach-like recreational water settings to endemic fungi, bacteria other than MRSA and *P. aeruginosa*, and other pathogens that are currently understudied and relating the microbial growth-based analysis to molecular quantification of relevant indicators of sand quality is also an interesting approach for the future of sand quality monitoring and beach management.

9.7 References

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Annex (List of Acronyms)

AMR – Anti-Microbial Resistance

ARG – Anti-microbial Resistance Genes

BWD – Bathing Water Directive

DNA – Deoxyribonucleic Acid

EU – European Union

ECDC – European Centre for Disease Control and prevention

FIB – Faecal Indicator Bacteria (Traditionally and in most parts of the world these bacteria are *Escherichia coli*, coliform bacteria, and enterococci)

FIO – Faecal Indicator Organism

GI – Gastrointestinal

MALDI-ToF – Matrix-Assisted Laser Desorption/Ionization-Time of Flight

MRSA – Methicillin-resistant *Staphylococcus aureus*

MST – Microbial Source Tracking

NGS – Next Generation Sequencing

OUT – Operational Taxonomic Units

PBS – Phosphate-Buffered Solution

PCR – Polymerase Chain Reaction

QMRA – Quantitative Microbial Risk Assessment

RWQC – Recreational Water Quality Criteria

Swash, vadose, intertidal zone – Beach area influenced by the wave activity

USA – United States of America

WHO – World Health Organisation

WSP – Water Safety Plan(s)