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Next-generation sequencing and culture-based techniques offer complementary insights into fungi and prokaryotes in beach sands

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ABSTRACT

A next-generation sequencing (NGS) approach, in conjunction with culture-based methods, was used to examine fungal and prokaryotic communities for the presence of potential pathogens in beach sands throughout Portugal. Culture-based fungal enumeration revealed low and variable concentrations of the species targeted (yeasts and dermatophytes), which were underrepresented in the community characterized by NGS targeting the ITS1 region. Conversely, NGS indicated that the potentially pathogenic species *Purpureocillium liliacinum* comprised nearly the entire fungal community. Culturable fecal indicator bacterial concentrations were low throughout the study and unrelated to communities characterized by NGS. Notably, the prokaryotic communities characterized revealed a considerable abundance of archaea. Results highlight differences in communities between methods in beach sand monitoring but indicate the techniques offer complementary insights. Thus, there is a need to leverage culture-based methods with NGS methods, using a toolbox approach, to determine appropriate targets and metrics for beach sand monitoring to adequately protect public health.

1. Introduction

Beaches consist of unconsolidated sediment, usually composed of sand, mud or pebbles. They are considered highly valued economic, recreational, and ecological areas (World Health Organization, 2003). Nevertheless, these environments also represent a reservoir for potentially pathogenic microorganisms (Pereira et al., 2013), not only because recreational water generally contains a mixture of pathogenic and non-pathogenic microbes, but also because tidally-wetted sands serve as a matrix for the development of microorganisms. This is in large part due to their relatively high organic matter content and relative humidity (Abdallah et al., 2005; Romão et al., 2015). Pathogens present on beaches can have different origins, but the majority likely come from exogenous sources, normally closely related to human activities and wild life (Heaney et al., 2009; Halliday and Gast, 2011). Pathogenic microorganisms that have previously been identified include: *Vibrio vulnificus* (Abdelzaher et al., 2010; Shah et al., 2011); *Salmonella* (Yamahara et al., 2012); *Campylobacter* (Yamahara et al., 2012); *Pseudomonas aeruginosa* (Esiobu et al., 2004); and *Staphylococcus aureus* (including MRSA strains) (Plano et al., 2013) among the bacteria, as well as the fungi *Aspergillus*, *Chrysosporium*, *Fusarium*, *Scedosporium*, *Scytalidium*, *Scopulariopsis* (Sabino et al., 2011), and *Candida* sp. (Shah et al., 2011). Pathogenic protozoans have also been found in sand, including *Giardia* and *Cryptosporidium* species (Abdelzaher et al., 2010), and nematode larvae and eggs (Shah et al., 2011).

While the World Health Organization has recognized sand as a reservoir and vector for infectious disease (World Health Organization, 2003), epidemiological studies are recent and have only begun to evaluate the risks of illness associated with beach sand and water contact. Several studies have suggested that there are clear links between sand quality and public health impacts (Sabino et al., 2014). More definitively, Heaney et al. (2012) reported a rise in gastrointestinal illness (GI) among beach users that had no contact with bathing water.

Contamination of water and sand with pathogens are intimately

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related, not only because most of the sources of pollution are common to both matrices, but also because tides, waves, and wind promote direct contact between both matrices (Pereira et al., 2013; Abreu et al., 2016; Vogel et al., 2016). Recreational waters are routinely monitored for microbiological quality, based on the presence and concentration of indicators of fecal contamination (Sabino et al., 2014), the fecal indicator bacteria (FIB) *Escherichia coli* and enterococci. The greater abundance of FIB in feces makes them easily targetable, since pathogens are less numerous and more difficult to detect, due in part to their great diversity (Solo-Gabriele et al., 2015). To date, however, these studies have only focused on bacteria and not fungi. It is estimated that contaminated coastal waters cause > 120 million cases of gastrointestinal illness and 50 million cases of respiratory disease worldwide each year (Halliday and Gast, 2011).

For detection and identification of microorganisms in sand and water, culture-based methods are typically used for screening of cultivable microorganisms, whereas molecular approaches are used for non-cultivable microorganisms. These molecular tools are inexpensive, relatively rapid, practical, and appear to correctly represent the microbial load found in sand. However, these methods only target a limited number of environmental microorganisms. Thus, the emergence of new molecular technologies and approaches, such as the use of high-throughput, next-generation sequencing (NGS), for the detection of sand-borne microorganisms has been considered in order to decrease limitations imposed by cultural-based methods and traditional molecular approaches (Staley et al., 2016). Furthermore, NGS allows for the possibility of evaluating relative abundances of a large majority of the microbiota present in a given sample.

Recently, Illumina-based NGS was used to characterize bacterial communities in beach sands throughout the northern hemisphere and it was found that beaches associated with the same bodies of water had highly similar taxonomic distributions of bacteria (Staley et al., 2016). Furthermore, moisture, presumably related to wave action, significantly influenced the bacterial diversity in beach sands in this study. Recent studies have also revealed that beach sands harbor less diverse bacterial communities when the water body is impaired (Halliday et al., 2014). A combination of qPCR and NGS approaches found that FIB decay more slowly in beach sands than in water (Zhang et al., 2015), further indicating that these bacteria are not useful to assess health risks in non-aquatic environments. Moreover, NGS has recently been used as part of a toolbox approach to assess sources of fecal contamination of recreational waters (Ahmed et al., 2015). This suggests that NGS-based methods may provide novel insights into potential health risks over traditional culture-based or molecular methodologies.

The goal of this paper was to evaluate the efficacy of high-throughput, NGS community analysis, compared to traditional methods, for the screening of microorganisms in beach sand. It is hypothesized that the NGS-based method would reveal a greater diversity of microorganisms compared to traditional methods due to the lack of a culture bias, leading to the attainment of more reliable data that can be applied in the appreciation of the microbiological quality of the beach sand. However, we expected that the same predominant taxa, present at high relative abundance, would be detected by both methods. Furthermore, traditional methods were anticipated to show greater sensitivity to specific low-abundance, potentially pathogenic taxa that may be missed by the NGS method. Taken together, results of this study expand the existing knowledge of microbial diversity in beach sands and offer a promising new tool for efficient detection and quantification of microorganisms in this important habitat.

2. Materials and methods

2.1. Sampling sites and methods

Sand samples were collected between April and November in 2013 from four regions in Portugal representing six recreational beaches (Fig. 1). For comparison, samples were also obtained from sandboxes from an urban center (Table 1). Samples were collected to a depth of approximately 10 cm using sterile gloves and placed into sterile plastic containers. All beach sand samples were collected from dry, backshore sand, as three equidistant sub-samples which were mixed into one composite (approximately 50 mL volume). The numbers of samples reported for culture-based and next-generation sequencing analyses varied, where culture-based analyses were typically performed on up to 10 samples collected from each region on each sample date. Next-generation sequence analysis was performed on duplicate DNA extracts (technical replicates) of the same homogenized preparations used for culture-based analysis, however, due to difficulties with amplification and rarefaction (see below), fewer samples were included in the final dataset. Samples were transported on ice to the laboratory and processed within 18 h of collection. Sand samples were not dehydrated prior to processing, in order to retain original characteristics; some degree of moisture is thus expected, as are any other eventual organic contaminants - further referred to as sand.

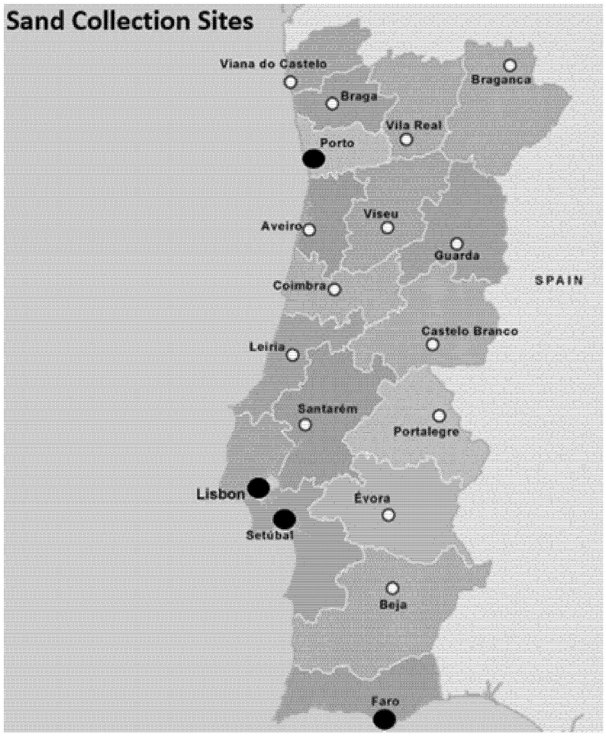

Fig. 1. Map of regions sampled.

Table 1
Description of sampling sites.

Region	Site name	Global positioning area
Algarve	Faro District	37N 8W
Alentejo	Setúbal District	37N 8W
North	Porto Disctrict Sites A, B and C	41N 8W
Lisbon	Sandbox	38N 9W

2.2. Enumeration of culturable fungi

Sample processing and culture-based fungal identification methods were performed as described by Sabino et al. (2011): samples (40 g) were diluted in 40 mL of sterilized distilled water, agitated for 30 min at 100 rpm and 0.2 mL of this suspension was spread-plated, in triplicate, onto Mycobiotic agar for dermatophytes, and malt extract agar (2%) with cloramphenicol (0.05 g L^{-1}), for non-dermatophyte