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Article

Beach Sand Contaminated by Dog Walking? A Molecular Case Study

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Abstract: Beach sand may act as a reservoir for numerous micro-organisms, including enteric pathogens. Several of these pathogens originate in human or animal feces, which may pose a public health risk. In August 2019, high levels of fecal indicator bacteria (FIB) were detected in the sand of the Azorean beach Prainha, Terceira Island, Portugal. Remediation measures were promptly implemented, including sand removal and the spraying of chlorine to restore the beach sand quality. To determine the biological source of the contamination, during the first campaign, supratidal sand samples were collected from several sites along the beach, followed by microbial source tracking (MST) analyses of *Bacteroides* markers for five animal species, including humans. Some of the sampling sites revealed the presence of marker genes from dogs, seagulls, and ruminants. Making use of the information on biological sources originating partially from dogs, the municipality enforced restrictive measures for dog-walking at the beach. Subsequent sampling campaigns detected low FIB contamination due to the mitigation and remediation measures that were undertaken, thereby no longer requiring MST marker-gene analysis. This is the first case study where the MST approach was used to determine the contamination sources in the supratidal sand of a coastal beach. Our results show that MST can be an essential approach to determine sources of fecal contamination in the sand. This study shows the importance of holistic management of beaches that should go beyond water quality monitoring for FIB, putting forth evidence for the need for sands also to be monitored.

Keywords: beach; coastal sand; fecal contamination; FIB; microbial source tracking (MST)

1. Introduction

Coastal areas provide a variety of recreational, athletic, and leisure activities, such as swimming, diving, water sports, and fishing [1]. However, many other activities do not require contact with water, as they take place on the sand or near the shoreline, such as running and walking along the seafront, practicing sand sports, children's activities like building sandcastles, and socializing and tanning [2-8]. The fascination with these coastal recreational environments tends to attract many users of all age groups and health conditions. This attraction ends up becoming an important local and often national economic revenue source.

Considering the relevance and frequent use of these spaces and recognizing that people spend more time on the sand than in water [1,8,9], it becomes evident that in

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addition to the need to maintain the quality of recreational waters, there is also a need to consider the quality of beach sands [10]. In a study conducted in the Azores [11], the authors raised some awareness about this issue, showing that sand can be a public health threat. That study investigated the origin of a skin-rash outbreak caused by sodium hypochlorite. Following a yearly cleaning of a restaurant bar and its toilet facilities, a degraded sewage distribution box leaked raw wastewater down the cliff, where the facilities were located. The fluid emerged in the sand, where the people were contaminated by direct contact with bare skin, resulting in the skin-rash outbreak.

Beach sand may act as a reservoir for various microorganisms, like enteric pathogens. The fecal pathogens potentially present in this environment tend to be considered a great risk to human health, especially for those with advanced age, diabetes, transient immunodepression, and respiratory problems [4,7,8,12]. Contact with contaminated sand either by a dermal route, ingestion, or accidental inhalation [3,4,7,11] can cause intestinal infections, verminoses and skin diseases, as well as exacerbate allergies, causing profound impacts on the population's life quality [5,8,13]. Many of these pathogens originate from human or animal feces [14]. When introduced into the environment, they can persist for long periods; even if their concentration is low, these pathogens can still be considered harmful to human health because some of the pathogens have low infectious doses [15]. Sand is no exception, as described in [16], where the fecal contamination indicators in sand remained viable for approximately three months following an extreme weather event in the island of Madeira.

In addition to possible contamination generated by contaminated seawater at times of rough seas or during high tides [12,17,18], beach sand can be contaminated by human or animal feces in several ways. Usually, contamination events arise from combined sewer overflow (CSO) or discharges of wastewater with inadequate or non-existing treatment. Animal feces can be classified as being from domestic or feral animals, with the most common ones being from dogs, cattle and other ruminants, some wild animals, and birds (like seagulls) [19-26].

Human feces or untreated wastewater tends to be classified as highly dangerous for human exposure. This statement is based on the idea that this type of feces has a greater potential for disease transmission to humans (greater compatibility and transmissibility of pathogens) compared to exposure to animal feces [27-31]. This ideology is based on the species barrier principle: each organism is more susceptible to its own set of disease-causing pathogens [32]. However, it should be noted that animal feces have a higher overall dominance, considering that these are usually less controlled than human ones, especially in rural places [33,34].

The contamination type can be classified as point source or diffuse. Point sources are easy to identify and manage, as the contamination reaches the watercourse in one concentrated place. Diffuse sources of contamination (also known as non-point) are usually more difficult to characterize and address, given their multiple contributions [6,18-20,35-38].

Despite the difficulties, the characterization of the contamination becomes crucial in management and remediation. Knowing the main sources of fecal contamination allows researchers and managing authorities to undertake minimization and remediation measures as well as identify the main risks to public health from these exposure routes [5,23].

Microbial source tracking (MST) is a molecular approach for tracking fecal contamination that aims to identify biological contamination sources. This approach facilitates the identifying the source of fecal contamination at the animal-species level, even in cases of diffuse origin [8,17,23,31,32,36,37,39-44]. This is based on the assumption that there are host-associated micro-organisms that each species has in their intestinal tract [17,19,20,23,32,35,36,45]. For example, *Bacteroidetes*, commensal bacteria prevalent in the intestinal microbiota of mammals, are mostly strict anaerobes adapted to living in the intestinal tract. These are one of the most selected bacteria for this approach because they have a cell density greater than 10^{10} /g of fecal matter [32]. They can exceed *Escherichia coli*

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(*E. coli*) concentrations, especially when associated with the human large intestine [32,46]. Additionally, they can easily be found in the environment, resulting from recent fecal contamination of water and sand [18,32]. Using specific gene markers for these bacteria, it is possible to identify the sources of fecal contamination present in the sand and subsequently adopt and implement corrective measures to minimize the risks for public health and the environment [36,37].

The objective of this case study is to identify and resolve the cause of an August of 2019 FIBs exceedance at Prainha Beach, in Terceira, Azores, Portugal, where a supratidal sand analysis revealed high levels of fecal indicator bacteria (FIB), of unknown origin. Currently, the threshold values used in Portugal for sand are 10 CFU/g of enterococci and 25 CFU/g of *E. coli* based on [47] and 100 coliform bacteria based on [2]. The beach was sampled in 11 sites to map the contamination and attempt to pinpoint the origin. Details on the mapping of samples are available in the materials and methods section. Several contamination origins were identified as potential contamination sources: a) run-off from mountains on the north coast, b) avian contamination by seagulls, c) direct beach contamination by users and dog walking, d) urban grey-waters infiltration via broken draining installations or storm-related overflow, and e) upwelling from the water table (which was tested locally for FIB but returned no useful information). Several remediation measures were undertaken to allow beach users to use the beach once more followed by the molecular analysis of the coastal sand samples to determine the likely sources of this contamination event.

2. Materials and Methods

Prainha beach is the only urban sandy beach in Angra do Heroísmo (GPS: 38°39'13.9"N 27°13'12.0"W). It is in the Bay of Angra and one of the main tourist sites, especially in the summer season. Visitors can swim on a beach located at the historic city center, which is a UNESCO world heritage site. The beach is bordered by the Bay of Angra, the marina, and the city wall. With the amount of hotel and restaurant services in the area, as well as the cultural offerings, it is a place of choice for residents and tourists, contributing to the economic growth of local agents. Tourist entertainment companies are located between Prainha and the marina in order to reach their target audience with greater accessibility. The whole area is frequented daily by approximately 300 to 400 users.

2.1. Collection and preservation of the sand samples

In August of 2019, there was an FIB exceedance in Prainha beach, with coliform bacteria ranging between 166 and >201 MPN/g, *E. coli* ranging between 28 and >201 MPN/g, and enterococci ranging between 10 and 201 MPN/g at different sampling sites of the beach. To identify the contamination dimension and origin, the sand was analyzed for standard microbiological contaminants (coliform bacteria, *E. coli* and enterococci) and by an MST approach. The sand samples were collected at several sites along the beach, according to the mapping displayed in Fig. 1 - 10 sites of supratidal sands and site 11 in the shoreline.

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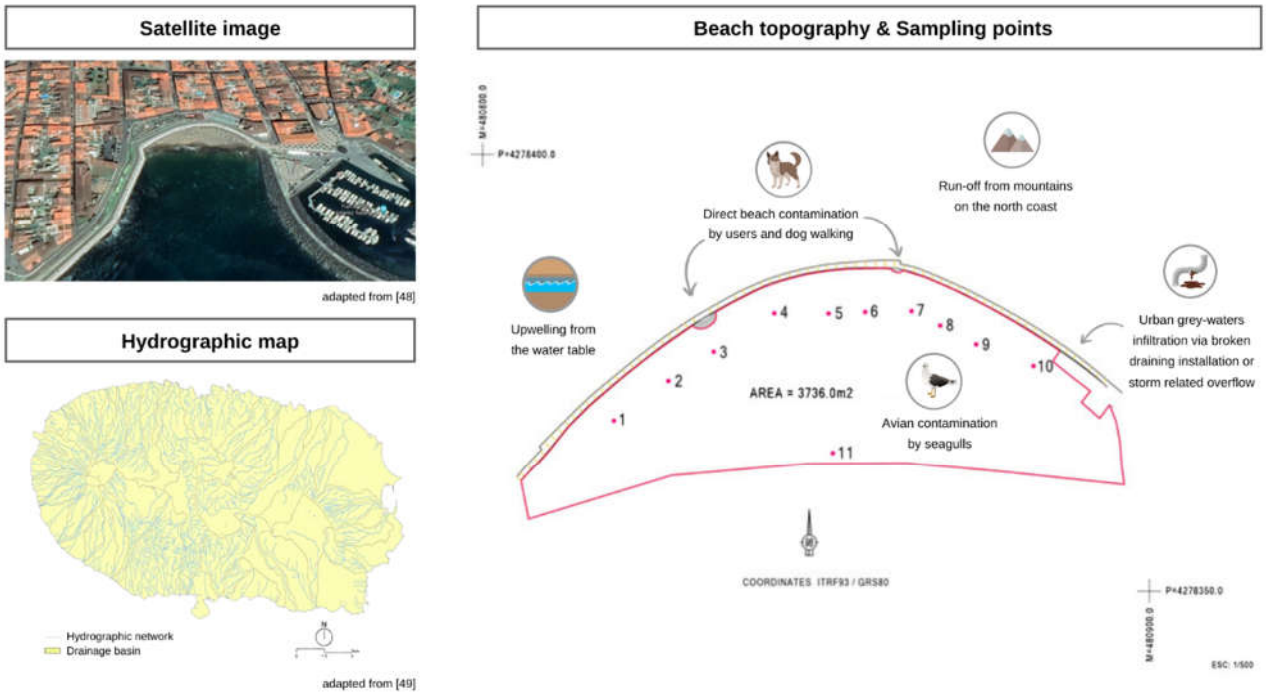

Figure 1. Prainha beach – satellite image and topography with an indication of the sampling sites 1 to 11. The hydrographic map of the island is also shown.

Sampling took place in depths of up to 10 cm from the surface into a sterile container, according to [2]. Samples were transported to the laboratory, protected from sunlight, and refrigerated. After homogenizing the sand, a part of each sample was analyzed immediately, and a portion was kept in a freezer at -20°C to allow posterior analyses. After the first sampling campaign, several interventions started to be applied to restore sand quality to allow beach use. The several sampling campaigns and management interventions are summarized in Figure 2.

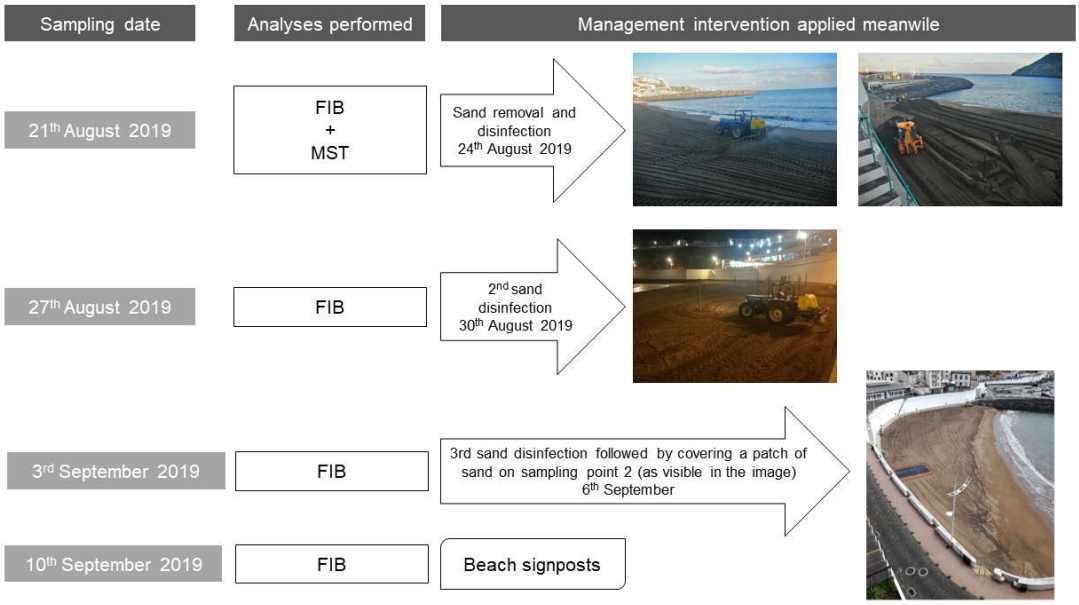

Figure 2. Sampling campaigns and interventions performed throughout the time.

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2.2. Microbiological parameters

For detection of the FIBs, we selected the methodology previously implemented by [47]: 50 g of sand from each sample were extracted in 500 mL of distilled water by shaking the mixture vertically with a rotation of 100 rpm for 30 mins followed by analyses using Quanti-Tray® systems from IDEXXTM (IDEXX, Westbrook, MN, USA) to determine enterococci most probable number (MPN) Enterolert® in 10 mL of the eluent, according to the manufacturer's instructions for water samples. The same procedure was applied for total coliforms and *E. coli* using the MPN Colilert® (also from IDEXXTM).

2.3. DNA Extraction from sand samples

Until the beginning of this work, there was still not and established standard or most suitable method for extracting genomic DNA from the microbes present in sand samples. Therefore, at the time of the contamination episode, the approach consisted of washing the sediments and successive filtration steps (PCR detection 1). Subsequently, while performing the geographical validation of MST, another approach was used that was based on the direct extraction of the DNA from the sand sample (frozen meanwhile) (PCR detection 2).

Approach A: A portion of each sand sample was used to extract the DNA. The first approach consisted of washing 20 g of sand with 50 mL of water, followed by 30 min of orbital agitation. The water was then filtered through 0.45 µm pore size polycarbonate membranes (diameter 47 mm) (Whatman®, Maidstone, UK), and the DNA was extracted from the filter membranes using an Aquadien (BioRad, California, USA) kit, using the manufacturer's protocol.

Approach B: This approach was used to extract the DNA from the frozen sand samples. Briefly, the DNA was extracted from 3 g of each sand sample using DNeasy Power Water Kit (Qiagen, Hilden, Germany), with some adaptations to increase the DNA yield (direct use of sand, instead of the membrane, and 10 min vortex of the sample, instead of the usual 5 min vortexing). Notes: 1) The DNeasy Power Water Kit was first used according to the manufacturer recommendations and then subjected to slight modifications, namely the amount of sand and the vortexing time. 2) This approach was also subsequently applied to fresh sand samples with success, for which 1.5 g of sand was enough (data not shown).

2.4. Microbial source tracking (MST) analyses

Primers used in this study were selected from the literature (Table 1). All the PCR conditions were tested and optimized, and all the primers were validated using local fecal samples of all five biological sources tested. This quality assurance was done on the premise that a geographical validation of MST primers is recommended before use [40,50].

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Table 1. List of microbial source tracking primers used and their respective annealing temperatures.

Target	Primers names	Sequence (5′-3′)	Conventional PCR annealing temperature	Tested by probe-based qPCR	References
Humans	HF183F BacR287	F: ATCATGAGTTCACATGTCCG R: CTTCCTCTCAGAACCCCTATCC P: FAM–CTAATGGAACGCATCCC–MGBEQ	53 °C	YES	[51]
Dogs	DF113F DF472R	F: ATCTCAAGAGCACATGCAA R: AATAAATCCGGATAACGCTC	53 °C	NO	[21]
Gulls	Gull-2F Gull-2R	F: TGCATCGACCTAAAGTTTTGAG R: GTCAAAGAGCGAGCAGTTACTA	53 °C	NO	[52]
Ruminants	RUM_CF128F RUM_Bac708R	F: CCAACYTTCCCGWTACTC R: CAATCGGAGTTCTTCGTG	60 °C	NO	[46]
Cows	CowM2F CowM2R	F: CGGCCAAATACTCCTGATCGT R: GCTTGTTGCGTTCCTTGAGATAAT P: FAM–AGGCACCTATGTCCTTTACCTCATCAACTACAGACA–MGBEQ	ND	YES	[53]
Cows	CowM3F CowM3R	F: CCTCTAATGGAAAATGGATGG-TATCT R: CCATACTTCGCCTGCTAATACCTT	53 °C	NO	[53]
Pigs	Bac41F Bac163R	F: GCATGAATTTAGCTTGCTAAATTT-GAT R: ACCTCATACGGTATTAATCCGC	60 °C	NO	[22]

Legend: ND: not determined for this approach. Note: The bovine primers were used only when samples: 1) were positive for ruminants (using conventional PCR) or 2) those analyzed by qPCR.

Each primer set (Table 1) was validated by conventional PCR for sensitivity and specificity. For that purpose, fresh fecal samples were collected from different regions of Portugal and selected from various individuals from different locations. This was done because the geographical location and individual characteristics might affect the composition of the gut flora and, in turn, the presence of enteric bacteria associated with MST markers. The fecal samples used in this study belonged to humans (part of the team incorporating this study), domestic animals (dogs and cats), livestock (donkeys, horses, cows, goats, sheep, and pigs) and birds (canaries, gulls, chickens, ducks, and turkeys). A total of 36 fecal samples were used: three belonged to humans; eight to domestic animals (four from dogs and four from cats); eighteen from livestock (one from donkeys, five from horses, five from cows, three from goats, two from sheep, and two from pigs); and seven from birds (one from canaries, two from seagulls, two from chickens, one from ducks, and one from turkeys).

DNA extraction from fecal samples was performed using commercial kits, such as the DNeasy PowerWater Kit (14900-100-NF) from Qiagen. The protocol associated with the Qiagen kit was adapted: instead of working with filters, a small amount of the fecal sample of interest was placed directly into the PowerWater DNA Bead Tube, together with 1 mL of the PW1 solution. After extraction, a NanoDrop One (Thermo Fisher Scientific) was used to verify and evaluate the quality and quantity of DNA obtained.

The volume of DNA used was determined based on its concentration. Usually, 2 µL of DNA was enough to obtain 20 ng to 30 ng of DNA per PCR reaction. However, in the case of DNAs with a lower concentration, 3 µL were added to reach the 20 ng to 30 ng of DNA per PCR reaction.

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2.4.1. PCR detection 1 – Fresh coastal sand samples

At the time of this contamination event, we had available and validated human and cow molecular markers from previous work [54]. Following the first DNA extraction method from sand, samples were tested by qPCR for human and cow contamination using the conditions previously described in [54]. Briefly, for the HF183/Bac287 Real-Time qPCR assay, 10 µL reaction mixtures containing 1x SsoAdvanced Universal Probe Supermix (Biorad, France), 0.2 mg/mL BSA (bovine serum albumin), 0.5 µM of each primer, 80 nM (FAM)-labelled probe and 2 µL of DNA template or molecular-grade water (no-template control - NTC) were prepared. The thermal cycling conditions were 10 min at 95ºC followed by 40 cycles of 15 s at 95ºC and 60 s at 60ºC [51]. For the CowM2 qPCR assay, 10 µL reaction mixtures containing 1x SsoAdvanced Univ Probes Supermix (Biorad, France), 0.2 mg/mL BSA, 1 µM of each primer, 80 nM (FAM)-labelled probe and 2 µL of DNA template or molecular-grade water (NTC) were prepared. The thermal cycling conditions were 10 min at 95ºC followed by 40 cycles of 15 s at 95ºC and 60 s at 60ºC [53].

2.4.2. PCR detection 2 - Frozen coastal sand samples

Meanwhile, more molecular markers were geographically validated (Table 1) and a new analysis was performed in the previously collected samples that had been kept at -20ºC. DNA obtained from the frozen sand samples (approach B) was analyzed with the primers indicated in Table 1 by conventional PCR. PCR reactions of 25 µL PCR was composed of 1x PCR buffer (BIOTAQ DNA polymerase), 1 U of Taq polymerase (BIOTAQ DNA polymerase), 3 mM of MgCl2, 1 mM of dNTPs, 1 µM of each primer pair, and 20-30 µg of DNA template.

PCR negative controls, on which no sample DNA was added, were included in each set of PCR reactions to monitor for contamination. Simultaneously, positive controls were also added.

The amplification occurred in a TPersonal thermal cycler (Biometra). PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min; 40 cycles of 1) denaturation step at 94°C for 45 s, 2) annealing step for each primers pairs, at temperatures indicated in Table 1 for 45 s, and 3) elongation step at 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were visualized using 1% agarose gels, stained with GelRed® Nucleic Acid Stain (Biotium), and using a 100 bp DNA ladder (PanReac AppliChem, ITW Reagents). The visualization of the gel was performed under a UV-light transilluminator (UVITEC Cambridge).

3. Results

3.1. Management and remediation measures

A supratidal sand sampling campaign that took place on August 21, 2020, of the Azorean beach Prainha resulted in the detection of high levels of FIB in 9 of the 10 sampling sites tested along the beach (Table 2, column "Fecal Indicator Bacteria results" and cells with bold text under). The samples exceeding the local standards used in sand monitoring underwent MST analyses. In order to protect the health of the beach users, three days before the following sampling campaign (August 24), a drastic intervention took place with the removal of 400 m^3 of sand and spraying of the entire beach (3736 m^2) with 1000 L of chlorine (6.5% hypochlorite aqueous solution). The second sampling campaign that took place on August 27, where a wet sand sample was also analyzed (sampling site 11), did not show any high levels of FIB. Only a low count (4 MPN/g) for coliforms in sampling site 2 suggested a possible new contamination after the beach sand treatment.

One week later (September 3 sampling campaign), sampling sites 1 to 10 were tested once again following a spraying event (August 30) with 600 L of a 6.5% hypochlorite solution along the entire length of the beach. The FIB results were below the recommendations used locally (10 MPN/g, 25 MPN/g and 100 MPN/g, of enterococci, *E. coli* and coliform bacteria, respectively) for the sampling sites 1 and 3 to 10; however, site 2 exceeded

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the recommendation for coliforms. This last result supported the previous suspicion that a de novo contamination was taking place after the beach spraying.

Unable to resolve the recurrent sand contamination immediately, and to distinguish fecal deposits from contamination upwelling, the solution was to physically eliminate the possible contamination sources by covering a 4m x 4m area (at sampling site 2) of the beach with an impermeable plastic film, as shown in Figure 2 (dark grey patch on the sand). Moreover, access to the beach was restricted to differentiate upwelling from deposition. A new spraying event took place on the entire beach length with 400 L of chlorine (6.5% hypochlorite aqueous solution) September 6. The September 10 sampling campaign (at sampling sites 2, 3, 4, 9 and 10) excluded a possible upwelling origin of the contamination. The local authorities assumed that the contamination could come from dog walking, after regular beach use hours, and a decision to discourage that activity with the local inhabitants was made and signage prohibiting dog walking was put in place.

3.2. MST markers validation

Until the beginning of this work, a standard or the most suitable method had not been established to extract genomic DNA of the microbes present in sand samples. Therefore, at the time of the contamination episode, the approach followed consisted of washing the sand samples followed by filtration and subsequent use of a DNA extraction kit (Approach A). Subsequently, while performing the geographical validation of more MST markers, another approach was used based on the direct extraction of the DNA from the sand samples (frozen meanwhile) (Approach B).

We verified that a higher yield and quality of the extracted DNA was obtained with Approach B, which granted more confidence in the results obtained by PCR. This approach was later applied to fresh sand samples with success, for which we verified that 1.5 g of sand was enough to obtain a good yield.

Geographic validation of the selected MST markers was performed using DNA from local fecal samples to avoid errors in the interpretation of results associated with an MST study, such as cases of cross-reactivity. The quality control results are summarized in Table 3. We observed that all primers tested were successfully validated. There was only one case of cross-reactivity detected, which referred to the DNA sample extracted from a fresh fecal sample from a horse, which was amplified with the ruminants primer set.

3.3. Microbiological and MST results

As already mentioned above, the microbiological parameters for the threshold limits currently used in Portugal for sand are 10 CFU/g of enterococci and 25 CFU/g of *E. coli* based on [47] and 100 coliform bacteria based on [2].

Analyzing Table 2, it is noticeable that in the samples collected on August 21, all the samples, except for sampling site 1, showed at least one FIB parameter higher than what is recommended (highlighted in Table 2). The following sampling campaign on September 3 also indicated fecal contamination in one of the sampling sites (highlighted in Table 2), and some other sampling sites showed FIBs (sites 3, 4, and 10). The final campaign that took place on September 10, 2019, did not indicate any FIB contamination.

Regarding the MST approach, the Dogs' marker gene was detected only on sampling location 4; the Gulls-2 marker gene was detected in sampling location 5, and a positive signal for the ruminants' marker gene was detected in sampling locations 8 and 9. No consistency was thus found in terms of contamination sources for the sites tested. Figure 2 shows the location of the sites that were positive for the different MST markers.

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Table 2: Results of the microbiological tests and Microbial source tracking (MST) of the sand samples collected on several dates throughout this study.

Sampling Date	**21st August 2019**						**27th August 2019**	**3rd September 2019**	**10th September 2019**
Sampling sites	**Faecal Indicator Bacteria results**	**MST Results**					**Faecal Indicator Bacteria results**		
		Humans	**Dogs**	**Gulls**	**Ruminants**	**Bovine**			
Site 1	Coliform bacteria, 1 MPN/g *Escherichia coli*, 1 MPN/g *Enterococcus* spp., <1 MPN/g	NEG	NEG	NEG	NEG	ND		Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g	
Site 2	**Coliform bacteria, 201 MPN/g** ***Escherichia coli*, 84 MPN/g** ***Enterococcus* spp., 201 MPN/g**	NEG	NEG	NEG	NEG	NEG	Coliform bacteria, 4 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g	**Coliform bacteria, 102 MPN/g** *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g	Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g
Site 3	**Coliform bacteria, >201 MPN/g** ***Escherichia coli*, >201 MPN/g** ***Enterococcus* spp., 201 MPN/g**	NEG	NEG	NEG	NEG	ND		Coliform bacteria, 4 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g	Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g
Site 4	Coliform bacteria, 14 MPN/g *Escherichia coli*, 1 MPN/g ***Enterococcus* spp., 10 MPN/g**	NEG	**POS**	NEG	NEG	NEG		Coliform bacteria, 9 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., 9 MPN/g	Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g

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Site 5	**Coliform bacteria, >201 MPN/g** ***Escherichia coli*, 110 MPN/g** ***Enterococcus* spp., 74 MPN/g**	NEG	NEG	**POS**	NEG	ND	Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g	Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g	
Site 6	**Coliform bacteria, 166 MPN/g** ***Escherichia coli*, 63 MPN/g** ***Enterococcus* spp., 51 MPN/g**	NEG	NEG	NEG	NEG	ND		Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g	
Site 7	Coliform bacteria, 5 MPN/g *Escherichia coli*, 1 MPN/g ***Enterococcus* spp., 130 MPN/g**	NEG	NEG	NEG	NEG	NEG		Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g	
Site 8	**Coliform bacteria, >201 MPN/g** *Escherichia coli*, 12 MPN/g ***Enterococcus* spp., 24 MPN/g**	NEG	NEG	NEG	**POS**	NEG		Coliform bacteria, 1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., 1 MPN/g	
Site 9	**Coliform bacteria, >201 MPN/g** ***Escherichia coli*, 28 MPN/g** ***Enterococcus* spp., 28 MPN/g**	NEG	NEG	NEG	**POS**	NEG	Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g	Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g	Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g
Site 10	**Coliform bacteria, 201 MPN/g** *Escherichia coli*, 13 MPN/g *Enterococcus* spp., 4 MPN/g	NEG	NEG	NEG	NEG	ND		Coliform bacteria, 2 MPN/g *Escherichia coli*, 1 MPN/g *Enterococcus* spp., <1 MPN/g	Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g

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Site 11							Coliform bacteria, <1 MPN/g *Escherichia coli*, <1 MPN/g *Enterococcus* spp., <1 MPN/g		

Legend: NEG – negative result (absence of DNA from the tested source); POS – positive result (detection of the DNA from the tested source). ND – Not determined.

Table 3. - Summarized table of the amplifications associated with each molecular marker selected. Represented with a "✓" are the samples that, when amplified by the marker, presented a band in the region of interest, and represented with a "✗" those that did not present bands in that region. ND – Not determined for the primers. The symbol "⚠" indicates that there was evidence of cross-reactivity when the marker was tested for a given faecal sample.

Target	Primers Names	Humans	Domestic Animals		Livestock						Birds			
			Cat	Dog	Donkey	Horse	Cow	Goat	Sheep	Pig	Canaries	Seagull	Chicken	Duck	Turkey
Humans	HF183F BacR287	✓	ND	✗	✗	✗	✗	✗	✗	✗	ND	✗	✗	✗	✗
Dogs	DF113F DF472R	✗	✗	✓	ND	✗	✗	✗	✗	✗	✗	ND	✗	ND	ND
Gulls	Gull-2F Gull-2R	✗	ND	✗	ND	✗	✗	✗	✗	✗	✗	✓	✗	✗	✗
Ruminants	RUM_CF128F RUM_Bac708R	✗	ND	✗	✗	⚠	✓	✓	✓	✗	ND	ND	✗	ND	ND
Cows	CowM3F CowM3R	✗	ND	✗	✗	✗	✓	✗	✗	✗	ND	ND	ND	✗	ND
Pigs	Bac41F Bac163R	✗	ND	✗	✗	✗	✗	✗	✗	✓	ND	ND	✗	ND	ND

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4. Discussion

Bacteriological analysis in sand has a similar objective to FIB enumeration in water, as it aims to detect and quantify bacteria (total coliforms, *E. coli*, and enterococci). Other bacterial and non-bacterial parameters may be used to indicate other forms of contamination, such as skin and hair shedding, and endemic species of interest in public health protection [8]. Fecal contamination, however, is the best-studied aspect in sand quality because it may not only represent a direct exposure route but also a diffuse source of contamination in water quality [10, 55].

It is important to carefully monitor the quality of the sand being tested and not just the water to avoid outbreaks like the one described in [11]. In that episode, a deteriorated sewage distribution box was the cause of an outbreak of 30 people, in which sand was the proven fomite. Events like this were the basis of a study [56], where an epidemiological study compared the health effects of handling sand in a biased scenario of a beach with a nearby, publicly owned, treatment-works outfall. In that study, handling the beach sand contaminated with *Enterococcus* led to an increase by over two-fold of gastrointestinal illness cases, with the highest incidence being in those who buried themselves in sand, with 3.3 times over more cases of GI illness than those of the reference group.

It seems we need to be more assertive in assessing the quality of a beach that is used by all kinds of people, including toddlers, who play with sand, including the elderly and immunologically compromised individuals (transiently or permanently), as well as those with cystic fibrosis or diabetics.

It may also be time to bring in climate change impacts on fecal contamination of unknown origin.

After reviewing all the information that is summarized in table 2 and from the remediation and management measures, it was possible to assign the respective contamination source locations (Fig. 3). The Dogs' markers at site 4 support the authorities' suspicions and reinforce the need to avoid this kind of practice that contributes to beach contamination. The Gulls' marker detection was not a surprise, given the sites being sampled.

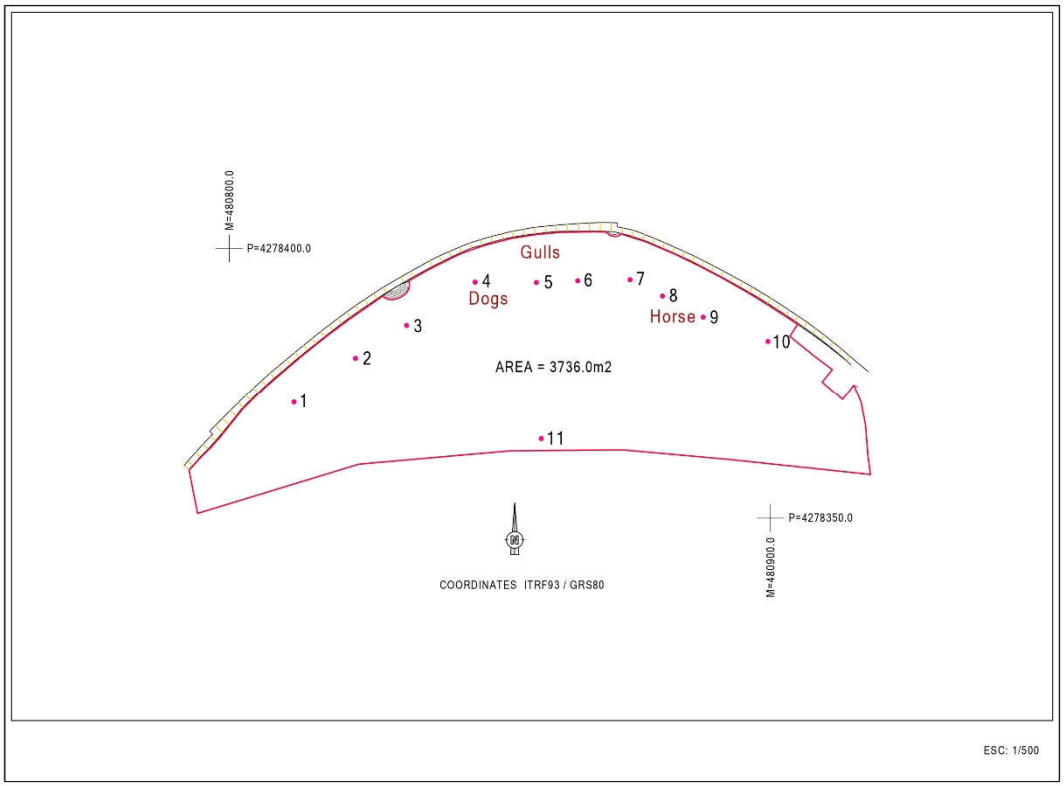

Figure 3. Assignment of the respective contamination sources to each of the sampled sites.

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As mentioned in the results, the primers used for the ruminants' marker gene gave a positive result in sampling locations 8 and 9. However, the Cows' marker gene (one of the possible ruminants) was not detected. In literature, there is a reference that mentions that the primers here used for the ruminants' marker gene may have cross-reactivity with the horses' [19,40,57-59]. Keeping this in mind, the team looked for the possible cultural events that had taken place near the beach at that time and discovered that throughout the summer, a horse-drawn carriage for sightseeing had been parked for some time near sites 8 and 9.

Enterococcus, *E. coli* and *Bacteroides* are three types of bacteria, which belong to different phyla [60], that are usually found in the intestines of warm-blooded animals [61]. Therefore, after a recent episode of fecal contamination, one can expect to find them all. However, the results obtained here highlight that despite the microbiological parameters being used above, in some cases, the DNA from *Bacteroides* was not detected, thus not allowing the assignment of the fecal contamination source. These results must be examined carefully, keeping in mind several factors: 1) we may not have used the most adequate primers to identify the DNA region present; 2) there might be other contamination sources besides the ones examined, e.g. from rodents; 3) the survival time of *Bacteroides* outside the intestine is much shorter (ca. 48 hours) when compared to *Enterococcus* (several weeks) or *E. coli* (6 hours) [62]. In this type of study, it is more probable that the last reason (the survival time) is behind the results obtained.

The several steps that were taken to decontaminate the sand were showed to be efficient. The detection of the horse- and dog-associated markers highlight the need to protect beaches from animal walking, which contributes to the high levels of FIBs subsequently detected. Moreover, further implementation of dog-walk restrictions and advisory signs helped to mitigate the contamination problem.

Although researchers sometimes use the traditional fecal indicators *E. coli* and *Enterococcus* to monitor sand quality [63-65], this methodology does not allow the biological source of the contamination to be inferred.

In the study [18] they analyzed intertidal sand, sediment, and overlying water at three shoreline sites and two associated rivers along an extended freshwater shoreline. The parameters analyzed were FIB, two MST markers (Gull2 and HF183), and the targeted metagenomic 16S rRNA gene. They were able to establish a relationship between bacteria in the sand, sediment, and overlying water; they concluded that FIB and MST markers were effective estimates of short-term conditions at these locations, while bacterial communities in sand and sediment reflected longer-term conditions.

The new WHO Guidelines for recreational water quality were released last July [8], recommending sand analysis for enterococci and fungi, with respectively provisional limits of 60 CFU/g and a guiding value of 89 CFU/g. The next natural step is for these recommendations to slowly be integrated into regulations; MST provides a valuable tool to investigate potential fecal pollution sources of both sand and water.

5. Conclusions

This paper examines the origin of the sand contamination of a beach in the Azores. This is a topic that needs to be addressed for sand-quality regulations. There are not many episodes reported, nor is there any formal legislation based on FIB.

This study shows that the microbial source tracking (MST) approach revealed itself as a useful method in determining the biological source of the fecal contamination, demonstrating that, in this case, it was attributed to multiple sources.

Another important and practical conclusion is the documentation of disinfection practices at the beach to lower the levels of fecal-indicator microbes in the sand.

Recognizing that beaches suffer contamination events, this study shows the importance of holistic management of the beaches worldwide that should go beyond the already established water-quality monitoring for FIB, putting forth evidence for the need for sands also to be monitored to avoid public health problems.

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References

1. Pinto, A.; Oliveira, A. Diversidade de microrganismos indicadores utilizados na avaliação da contaminação fecal de areias de praias recreacionais marinhas: Estado atual do conhecimento e perspectivas. *Mundo Saúde* 2011, *35*, 105-114; DOI:10.15343/0104-7809.2011351105114.
2. Brandão, J.; Wergikosky, B.; Rosado, C.; Noronha, G.; Veríssimo, C.; Falcão, L.; Giraldes, A.; Simões, M.; Rebelo, H. *Qualidade Microbiológica de areias de Praias Litorais – Relatório Final*; Associação Bandeira Azul da Europa: Alfragide, Portugal, 2002; pp. 1-132.
3. Brandão, J.; Silva, C.; Alves, C.; Cunha, M.; Moura, I.; Veríssimo, C.; Wergikoski, B.; Parada, H.; Falcão, L.; Barroso, M.; Rodrigues, R.; Sabino, R.; Rosado, L. *Monitorização da qualidade das areias em zonas balneares – Época balnear de 2008*; Associação Bandeira Azul da Europa: Lisboa, Portugal, 2008; pp. 1-35.
4. Brito, S.; Sabino, R.; Veríssimo, C.; Silva, S.; Valério, E.; Brandão, J. Fungos em areias e águas costeiras e interiores em Portugal – Relevância para a saúde humana e bem-estar. *Observações_ Boletim Epidemiológico* 2019, 11, 49-54.
5. Lescreck, M.; Petroni, R.; Cortez, F.; Santos, A.; Coutinho, P.; Pusceddu, F. Análise da qualidade sanitária da areia das praias de Santos, litoral do estado de São Paulo. *Eng Sanit Ambient* 2016, *21*, 777-782; DOI:10.1590/s1413-41522016149550.
6. Safaie, A.; Weiskerger, C.; Nevers, M.; Byappanahalli, M.; Phanikumar, M. Evaluating the impacts of foreshore sand and birds on microbiological contamination at a freshwater beach. *Water Res* 2021, *190*, 116671; DOI:10.1016/j.watres.2020.116671.
7. Weiskerger, C.; Brandão, J. Fungal contaminants in water and sand: A new frontier for quantitative microbial risk assessment. *Curr Opin Environ Sci Health* 2020, *16*, 73-81; DOI:10.1016/j.coesh.2020.03.001.
8. World Health Organization. *Guidelines on recreational water quality: Volume 1 coastal and fresh waters*; World Health Organization: Geneva, Switzerland, 2021; pp. 1-164.
9. World Health Organization. *Guidelines for safe recreational water environments: Volume 1 coastal and fresh waters*; World Health Organization: Geneva, Switzerland, 2003; pp. 1-219.
10. Solo-Gabriele, H.; Harwood, V.; Kay, D.; Fujioka, R.; Sadowsky, M.; Whitman, R.; Wither, A.; Caniça, M.; Carvalho da Fonseca, R.; Duarte, A.; Edge, T.; Gargaté, M.; Gunde-Cimerman, N.; Hagen, F.; McLellan, S.; Nogueira da Silva, A.; Novak Babič, M.; Prada, S.; Rodrigues, R.; Romão, D.; Sabino, R.; Samson, R.; Segal, E.; Staley, C.; Taylor, H.; Veríssimo, C.; Viegas, C.; Barroso, H.; Brandão, J. Beach sand and the potential for infectious disease transmission: Observations and recommendations. *J Mar Biolog Assoc U.K.* 2015, *96*, 101-120; DOI:10.1017/s0025315415000843.
11. Brandão, J.; Albergaria, I.; Albuquerque, J.; José, S.; Grossinho, J.; Ferreira, F.; Raposo, A.; Rodrigues, R.; Silva, C.; Jordao, L.; Sousa, M.; Rebelo, M.; Veríssimo, C.; Sabino, R.; Amaro, T.; Cardoso, F.; Patrão-Costa, M.; Solo-Gabriele, H. Untreated sewage contamination of beach sand from a leaking underground sewage system. *Sci Total Environ* 2020, *740*, 140237; DOI:10.1016/j.scitotenv.2020.140237.
12. Gast, R.; Gorrell, L.; Raubenheimer, B.; Elgar, S. Impact of erosion and accretion on the distribution of enterococci in beach sands. *Cont Shelf Res* 2011, *31*, 1457-1461; DOI:10.1016/j.csr.2011.06.011.
13. Brandão, J.; Gangneux, J.; Arikan-Akdagli, S.; Barac, A.; Bostanaru, A.; Brito, S.; Bull, M.; Çerikçioğlu, N.; Chapman, B.; Efstratiou, M.; Ergin, Ç.; Frenkel, M.; Gitto, A.; Gonçalves, C.; Guégan, H.; Gunde-Cimerman, N.; Güran, M.; Irinyi, L.; Jonikaitė, E.; Kataržytė, M.; Klingspor, L.; Mares, M.; Meijer, W.; Melchers, W.; Meletiadis, J.; Meyer, W.; Nastasa, V.; Babič, M.; Ogunc, D.; Ozhak, B.; Prigitano, A.; Ranque, S.; Rusu, R.; Sabino, R.; Sampaio, A.; Silva, S.; Stephens, J.; Tehupeiory-Kooreman, M.; Tortorano, A.; Velegraki, A.; Veríssimo, C.; Wunderlich, G.; Segal, E. Mycosands: Fungal diversity and abundance in beach sand and recreational waters — Relevance to human health. *Sci Total Environ* 2021, *781*, 146598; DOI:10.1016/j.scitotenv.2021.146598.
14. Ko, H.; Cho, K.; Park, S.; Kim, J.; Kang, J.; Jeong, Y.; Choi, J.; Sin, Y.; Lee, C.; Ko, G. Host-specific Bacteroides markers-based microbial source tracking in aquaculture areas. *Microbes Environ* 2018, *33*, 151-161; DOI:10.1264/jsme2.ME17166.
15. Pathogen safety data sheets. Available online: https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/pathogen-safety-data-sheets-risk-assessment.html (accessed on Apr 20, 2022).
16. Romão, D.; Abreu, R.; Calado, G.; Freitas, F.; Rodrigues, P.; Ferreira, C.; Campos, A.; Temtem, R.; Freitas, M.; Andrade, C.; Prada, S.; Figueira, C.; Brandão, J. Madeira 2010 - Aftermath of flashfloods and mudslides on bathing water quality indicators

and on sand microbial levels. In Pan-European Symposium on Water and Sanitation Safety Planning and Extreme Weather Events, Bilthoven, Netherlands (Apr 7, 2017); DOI:10.13140/RG.2.2.27784.44802.

17. Henry, R.; Schang, C.; Coutts, S.; Kolotelo, P.; Prosser, T.; Crosbie, N.; Grant, T.; Cottam, D.; O'Brien, P.; Deletic, A.; McCarthy, D. Into the deep: Evaluation of SourceTracker for assessment of faecal contamination of coastal waters. *Water Res* 2016, *93*, 242-253; DOI:10.1016/j.watres.2016.02.029.
18. Nevers, M.; Byappanahalli, M.; Nakatsu, C.; Kinzelman, J.; Phanikumar, M.; Shively, D.; Spoljaric, A. Interaction of bacterial communities and indicators of water quality in shoreline sand, sediment, and water of Lake Michigan. *Water Res* 2020, *178*, 115671; DOI:10.1016/j.watres.2020.115671.
19. Ballesté, E.; Demeter, K.; Masterson, B.; Timoneda, N.; Sala-Comorera, L.; Meijer, W. Implementation and integration of microbial source tracking in a river watershed monitoring plan. *Sci Total Environ* 2020, *736*, 139573; DOI:10.1016/j.scitotenv.2020.139573.
20. Gawler, A.; Beecher, J.; Brandão, J.; Carroll, N.; Falcão, L.; Gourmelon, M.; Masterson, B.; Nunes, B.; Porter, J.; Rincé, A.; Rodrigues, R.; Thorp, M.; Martin Walters, J.; Meijer, W. Validation of host-specific Bacteriodales 16S rRNA genes as markers to determine the origin of faecal pollution in Atlantic Rim countries of the European Union. *Water Res* 2007, *41*, 3780-3784; DOI:10.1016/j.watres.2007.01.028.
21. Hussein, K.; Waines, P.; Nisr, R.; Glegg, G.; Bradley, G. Development and use of Bacteroides 16S rRNA polymerase chain reaction assay for source tracking dog faecal pollution in bathing waters. *Hydrol Current Res* 2014, *5*, 1-8; DOI:10.4172/2157-7587.1000163.
22. Mieszkin, S.; Furet, J.; Corthier, G.; Gourmelon, M. Estimation of pig fecal contamination in a river catchment by Real-Time PCR using two pig-specific Bacteroidales 16S rRNA genetic markers. *Appl Environ Microbiol* 2009, *75*, 3045-3054; DOI:10.1128/aem.02343-08.
23. Nguyen, K.; Senay, C.; Young, S.; Nayak, B.; Lobos, A.; Conrad, J.; Harwood, V. Determination of wild animal sources of fecal indicator bacteria by microbial source tracking (MST) influences regulatory decisions. *Water Res* 2018, *144*, 424-434; DOI:10.1016/j.watres.2018.07.034.
24. Ryu, H.; Griffith, J.; Khan, I.; Hill, S.; Edge, T.; Toledo-Hernandez, C.; Gonzalez-Nieves, J.; Santo Domingo, J. Comparison of gull feces-specific assays targeting the 16S rRNA genes of Catellicoccus marimammalium and Streptococcus spp. *Appl Environ Microbiol* 2012, *78*, 1909-1916; DOI:10.1128/aem.07192-11.
25. Shanks, O.; White, K.; Kelty, C.; Hayes, S.; Sivaganesan, M.; Jenkins, M.; Varma, M.; Haugland, R. Performance assessment PCR-Based assays targeting Bacteroidales genetic markers of bovine fecal pollution. *Appl Environ Microbiol* 2010, *76*, 1359-1366; DOI:10.1128/aem.02033-09.
26. Xu, Y.; Li, Z.; Liu, R.; Liang, H.; Yu, Z.; Zhang, H. Validation of Bacteroidales-based microbial source tracking markers for pig fecal pollution and their application in two rivers of North China. *Front Environ Sci Eng* 2020, *14*, 1-8; DOI:10.1007/s11783-020-1246-9.
27. Schoen, M.; Ashbolt, N. Assessing pathogen risk to swimmers at non-sewage impacted recreational beaches. *Environ Sci Technol* 2010, *44*, 2286-2291; DOI:10.1021/es903523q.
28. Schoen, M.; Soller, J.; Ashbolt, N. Evaluating the importance of faecal sources in human-impacted waters. *Water Res* 2011, *45*, 2670-2680; DOI:10.1016/j.watres.2011.02.025.
29. Soller, J.; Bartrand, T.; Ashbolt, N.; Ravenscroft, J.; Wade, T. Estimating the primary etiologic agents in recreational freshwaters impacted by human sources of faecal contamination. *Water Res* 2010, *44*, 4736-4747; DOI:10.1016/j.watres.2010.07.064.
30. Soller, J.; Schoen, M.; Bartrand, T.; Ravenscroft, J.; Ashbolt, N. Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination. *Water Res* 2010, *44*, 4674-4691; DOI:10.1016/j.watres.2010.06.049.
31. Zhang, Q.; Gallard, J.; Wu, B.; Harwood, V.; Sadowsky, M.; Hamilton, K.; Ahmed, W. Synergy between quantitative microbial source tracking (qMST) and quantitative microbial risk assessment (QMRA): A review and prospectus. *Environ Int* 2019, *130*, 104703; DOI:10.1016/j.envint.2019.03.051.
32. Fujioka, R.; Solo-Gabriele, H.; Byappanahalli, M.; Kirs, M. U.S. Recreational Water Quality Criteria: A vision for the future. *Int J Environ Res Public Health* 2015, *12*, 7752-7776; DOI:10.3390/ijerph120707752.
33. Delahoy, M.; Wodnik, B.; McAliley, L.; Penakalapati, G.; Swarthout, J.; Freeman, M.; Levy, K. Pathogens transmitted in animal feces in low- and middle-income countries. *Int J Hyg Environ Health* 2018, *221*, 661-676; DOI:10.1016/j.ijheh.2018.03.005.
34. Penakalapati, G.; Swarthout, J.; Delahoy, M.; McAliley, L.; Wodnik, B.; Levy, K.; Freeman, M. Exposure to animal feces and human health: A systematic review and proposed research priorities. *Environ Sci Technol* 2017, *51*, 11537-11552; DOI:10.1021/acs.est.7b02811.
35. García-Aljaro, C.; Blanch, A.; Campos, C.; Jofre, J.; Lucena, F. Pathogens, faecal indicators and human-specific microbial source-tracking markers in sewage. *J Appl Microbiol* 2019, *126*, 701-717; DOI:10.1111/jam.14112.
36. Griffith, J.; Layton, B.; Boehm, A.; Holden, P.; Jay, J.; Hagedorn, C.; McGee, C.; Weisberg, S. *The California microbial source identification manual: A tiered approach to identifying fecal pollution sources to beaches*; Southern California Coastal Water Research Project (SCCWRP): Costa Mesa, United States of America, 2013; pp. 1-88.

37. Hughes, B.; Beale, D.; Dennis, P.; Cook, S.; Ahmed, W. Cross-comparison of human wastewater-associated molecular markers in relation to fecal indicator bacteria and enteric viruses in recreational beach waters. *Appl Environ Microbiol* 2017, *83*, e00028-17; DOI:10.1128/aem.00028-17.
38. Unno, T.; Staley, C.; Brown, C.; Han, D.; Sadowsky, M.; Hur, H. Fecal pollution: New trends and challenges in microbial source tracking using next-generation sequencing. *Environ Microbiol* 2018, *20*, 3132-3140; DOI:10.1111/1462-2920.14281.
39. Field, K.; Samadpour, M. Fecal source tracking, the indicator paradigm, and managing water quality. *Water Res* 2007, *41*, 3517-3538; DOI:10.1016/j.watres.2007.06.056.
40. Harwood, V.; Staley, C.; Badgley, B.; Borges, K.; Korajkic, A. Microbial source tracking markers for detection of fecal contamination in environmental waters: Relationships between pathogens and human health outcomes. *FEMS Microbiol Rev* 2014, *38*, 1-40; DOI:10.1111/1574-6976.12031.
41. Staley, Z.; Boyd, R.; Shum, P.; Edge, T. Microbial source tracking using quantitative and digital PCR to identify sources of fecal contamination in stormwater, river water, and beach water in a Great Lakes area of concern. *Appl Environ Microbiol* 2018, *84*, e01634-18; DOI:10.1128/aem.01634-18.
42. Stoeckel, D. Selection and application of microbial source tracking tools for water-quality investigations. In *Book 2: Collection of environmental data, Section A. Biological science - Techniques and Methods*; U.S. Geological Survey: Reston, Virginia, 2005; pp.1-43; DOI:10.3133/tm2a3.
43. U.S. Environmental Protection Agency; Office of Water; Office of Science and Technology; Health and Ecological Criteria Division. *Overview of technical support materials: A guide to the site-specific alternative recreational criteria TSM documents*; Environmental Protection Agency, 2014; pp. 1-20.
44. Vadde, K.; McCarthy, A.; Rong, R.; Sekar, R. Quantification of microbial source tracking and pathogenic bacterial markers in water and sediments of Tiaoxi River (Taihu Watershed). *Front Microbiol* 2019, *10*, 1-19; DOI:10.3389/fmicb.2019.00699.
45. Ahmed, W.; Gyawali, P.; Feng, S.; McLellan, S. Host specificity and sensitivity of established and novel sewage-associated marker genes in human and nonhuman fecal samples. *Appl Environ Microbiol* 2019, *85*, e00641-19; DOI:10.1128/aem.00641-19.
46. Bernhard, A.; Field, K. A PCR assay to discriminate human and ruminant feces on the basis of host differences in Bacteroides-Prevotella genes encoding 16S rRNA. *Appl Environ Microbiol* 2000, *66*, 4571-4574; DOI:10.1128/aem.66.10.4571-4574.2000.
47. Sabino, R.; Veríssimo, C.; Cunha, M.; Wergikoski, B.; Ferreira, F.; Rodrigues, R.; Parada, H.; Falcão, L.; Rosado, L.; Pinheiro, C.; Paixão, E.; Brandão, J. Pathogenic fungi: An unacknowledged risk at coastal resorts? New insights on microbiological sand quality in Portugal. *Mar Pollut Bull* 2011, *62*, 1506-1511; DOI:10.1016/j.marpolbul.2011.04.008.
48. Google Earth. Available online: https://www.google.com/intl/pt-PT/earth/ (accessed on Jan 21, 2021).
49. Simbiente Açores - Engenharia e Gestão Ambiental. Plano de gestão da região hidrográfica dos Açores 2022-2027 – Relatório Técnico: Volume 3. Terceira - Caracterização e diagnóstico da situação de referência; 2021; pp. 1-323.
50. Holcomb, D.; Knee, J.; Sumner, T.; Adriano, Z.; de Bruijn, E.; Nalá, R.; Cumming, O.; Brown, J.; Stewart, J. Human fecal contamination of water, soil, and surfaces in households sharing poor-quality sanitation facilities in Maputo, Mozambique. *Int J Hyg Environ Health* 2020, *226*, 113496; DOI:10.1016/j.ijheh.2020.113496.
51. Green, H.; Haugland, R.; Varma, M.; Millen, H.; Borchardt, M.; Field, K.; Walters, W.; Knight, R.; Sivaganesan, M.; Kelty, C.; Shanks, O. Improved HF183 Quantitative Real-Time PCR Assay for Characterization of Human Fecal Pollution in Ambient Surface Water Samples. *Appl Environ Microbiol* 2014, *80*, 3086-3094; DOI:10.1128/aem.04137-13.
52. Lu, J.; Santo Domingo, J.; Lamendella, R.; Edge, T.; Hill, S. Phylogenetic Diversity and Molecular Detection of Bacteria in Gull Feces. *Appl Environ Microbiol* 2008, *74*, 3969-3976; DOI:10.1128/aem.00019-08.
53. Shanks, O.; Atikovic, E.; Blackwood, A.; Lu, J.; Noble, R.; Domingo, J.; Seifring, S.; Sivaganesan, M.; Haugland, R. Quantitative PCR for Detection and Enumeration of Genetic Markers of Bovine Fecal Pollution. *Appl Environ Microbiol* 2008, *74*, 745-752; DOI:10.1128/aem.01843-07.
54. Teixeira, P.; Dias, D.; Costa, S.; Brown, B.; Silva, S.; Valério, E. Bacteroides spp. and traditional fecal indicator bacteria in water quality assessment – An integrated approach for hydric resources management in urban centers. *J Environ Manage* 2020, *271*, 110989; DOI:10.1016/j.jenvman.2020.110989.
55. Whitman, R.; Harwood, V.; Edge, T.; Nevers, M.; Byappanahalli, M.; Vijayavel, K.; Brandão, J.; Sadowsky, M.; Alm, E.; Crowe, A.; Ferguson, D.; Ge, Z.; Halliday, E.; Kinzelman, J.; Kleinheinz, G.; Przybyla-Kelly, K.; Staley, C.; Staley, Z.; Solo-Gabriele, H. Microbes in beach sands: Integrating environment, ecology and public health. *Rev Environ Sci Biotechnol* 2014, *13*, 329-368; DOI:10.1007/s11157-014-9340-8.
56. Heaney, C.; Sams, E.; Dufour, A.; Brenner, K.; Haugland, R.; Chern, E.; Wing, S.; Marshall, S.; Love, D.; Serre, M.; Noble, R.; Wade, T. Fecal indicators in sand, sand contact, and risk of enteric illness among beachgoers. *Epidemiology* 2012, *23*, 95-106; DOI:10.1097/ede.0b013e31823b504c.
57. Kirs, M.; Harwood, V.; Fidler, A.; Gillespie, P.; Fyfe, W.; Blackwood, A.; Cornelisen, C. Source tracking faecal contamination in an urbanised and a rural waterway in the Nelson-Tasman region, New Zealand. *N Z J Mar Freshwater Res* 2011, *45*, 43-58; DOI:10.1080/00288330.2010.535494.
58. Somnark, P.; Chyerochana, N.; Kongprajug, A.; Mongkolsuk, S.; Sirikanchana, K. PCR data and comparative performance of Bacteroidales microbial source tracking genetic markers. *Data Br* 2018, *19*, 156-169; DOI:10.1016/j.dib.2018.04.129.
59. Zhang, Y.; Wu, R.; Lin, K.; Wang, Y.; Lu, J. Performance of host-associated genetic markers for microbial source tracking in China. *Water Res* 2020, *175*, 115670; DOI:10.1016/j.watres.2020.115670.

60. Rinninella, E.; Raoul, P.; Cintoni, M.; Franceschi, F.; Miggiano, G.; Gasbarrini, A.; Mele, M. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms* 2019, *7*, 14; DOI:10.3390/microorganisms7010014.
61. Ashbolt, N.; Grabow, W.; Snozzi, M. Indicators of microbial water quality. In *Water quality: Guidelines, standards and health: Assessment of risk and risk management for water-related infectious disease*; Fewtrell, L., Bartram, J., Eds.; World Health Organization: London, UK, 2001; pp. 289-316.
62. Bolinger, H. The survival of enterococcus faecalis and bacteroides fragilis on four different food contact surfaces. Master of Science, Rutgers School of Graduate Studies University–New Brunswick, 2014; DOI:10.7282/T3GB22H6.
63. Elmanama, A.; Fahd, M.; Afifi, S.; Abdallah, S.; Bahr, S. Microbiological beach sand quality in Gaza Strip in comparison to seawater quality. *Environ Res* 2005, *99*, 1-10; DOI:10.1016/j.envres.2004.12.014.
64. Halliday, E.; Gast, R. Bacteria in Beach Sands: An Emerging Challenge in Protecting Coastal Water Quality and Bather Health. *Environ Sci Technol* 2011, *45*, 370-379; DOI:10.1021/es102747s.
65. Pereira, E.; Figueira, C.; Aguiar, N.; Vasconcelos, R.; Vasconcelos, S.; Calado, G.; Brandão, J.; Prada, S. Microbiological and mycological beach sand quality in a volcanic environment: Madeira archipelago, Portugal. *Sci Total Environ* 2013, *461-462*, 469-479; DOI:10.1016/j.scitotenv.2013.05.025.