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Research Article

Microbial communities in the water surface microlayer and associations with microbes in aerosols, beach sand, and bulk water

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Abstract

The water surface microlayer (SML) serves as a boundary through which microbes can be exchanged. To evaluate exchanges of microbes, this study compared microbial communities within different reservoirs, with an emphasis on the water SML and aerosols. Additionally, the microbial communities during a sewage spill and perigean tides were evaluated and the results were compared to times without these events. Results show that during perigean tides and during the sewage spill, levels of culturable bacteria were highest and showed an increase via sequencing in potential pathogenic bacteria (*Corynebacterium* and *Vibrio,* which increased from 3.5%-1800% depending on sample type). In the aerosol samples, *Corynebacterium* (average of 2.0%), *Vibrio* (1.6%), and *Staphylococcus* (10%), were the most abundant genera. Aerosolization factors, which were used to examine the transfer of the microbes, were high for these three genera. Measurements of general marine bacteria (GMB) by culture showed a weak but significant correlation between culturable GMB in aerosol samples versus in water and in the SML. More research is needed to evaluate the exchange of pathogens between the SML and air, given the increase in potentially pathogenic microbes within the SML during rare events and evidence that suggests that microbes maintain viability during transfers across reservoirs.

Keywords: aerosolization, enterococci, general marine bacteria, king tide, sewage spill, surface microlayer

Introduction

Coastal environments provide diverse microbial habitats and are known to harbor microbes of human health concern, ranging from fecal indicator organisms (indicating the potential presence of disease-causing microbes) to direct measures of pathogens including viruses, bacteria, fungi, and protozoa (Abdelzaher et al. 2011, Shibata and Solo-Gabriele 2012, Whitman et al. 2014, Motlagh and Yang 2019). The water surface microlayer, bulk water, beach sand and air are the major reservoirs for microbial communities within the beach. On their own they have each been extensively studied. Pathogens, specifically have also been found in water, beach sand, and air reservoirs at beaches. These pathogenic bacteria can affect the health of individuals that frequent the beach. There are mechanisms, including turbulence from breaking waves, that can facilitate microbial transfer and pathogen exchange among these reservoirs.

Exchange of microbes between the air and sea surface is mediated by the water surface microlayer (SML). The sea surface contains the microlayer, defined as the top millimeter of the ocean surface (Zäncker et al. 2018), which is physiochemically and biologically distinct from the subsurface water below (Marty et al. 1979; Carlucci et al. 1992; Cunliffe et al. 2013). Here marine aerosols are mainly produced from bubble bursting (Blanchard and Woodcock 1957, Blanchard and Syzdek 1972, Blanchard and Syzdek 1982). In brief, small air bubbles, which are formed by breaking waves/whitecaps, are entrained within the water column. As they begin to resurface hydrophobic material collects and accumulates on the outside of the bubble (Grammatika and Zimmerman 2001, Aller et al. 2005, Cunliffe et al. 2013). Once the bubbles reach the surface, the collected material is then ejected into the atmosphere, thus releasing particles including bacteria in the form of aerosols (de Leeuw et al. 2000, de Leeuw et al. 2011). Microbes can become concentrated in aerosols resulting in a potentially higher dose of exposure within the coastal environment.

As the human population continues to expand, the population is shifting towards the coast (Roberts and Hawkins 1999), which leads to intense coastal development and urbanization. Surface water contamination from sewage will likely increase (Weiskerger et al. 2019) through impending intensification of population along the coastline in the coming years (Brown et al. 2008). Climate change, specifically sea level rise, has drastically modified shorelines (Defeo et al. 2009) and may increase exposure to waterborne pathogens (Weiskerger et al. 2019). Over time sea level rise is raising the height of the tidal system and the average daily water levels are further increasing (US EPA 2022). Compounding sea level rise with perigean spring tides (king tide) results in high tides

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reaching higher and extending further inland (US EPA 2022). Climate change is also expected to increase the frequency of wastewater treatment plant failures and combined sewer overflow discharge to surface water during extreme rain events (Trtanj et al. 2016).

The interaction between all four reservoirs of beach microbes (water SML, bulk water, beach sand, and air) is rarely studied in one coastal location. Studies have hypothesized the interaction between bulk water, beach sand, and air (O'Mullan et al. 2017, Pendergraft et al. 2021). Very few studies provide experimental data in the nearshore environment to examine the transfer of microbes from the SML to bulk water, beach sand, and air. In one study from Graham et al. (2018), the interaction between the bulk water, beach sand and air was examined at three California beaches using molecular methods to document the microbial communities in bulk water and aerosols.

This study aimed to evaluate similarities and differences between the microbial communities within different reservoirs, with an emphasis on the water SML and aerosols. Given that multiple sampling events were conducted we were additionally able to evaluate the microbial community structure during a sewage spill and king tide events and compare the results to times without these events. Bacteria were analyzed using culture-based techniques to quantify viable general marine bacteria (GMB) and enterococci, and sequencing was used to characterize microbial communities. This study is unique in the sample collection design which included aerosols, beach sand, plus water samples collected from seven sub-environments, two of which include the SML (at knee and waist depth). The inclusion of the SML is important as it represents the interface between bulk water, beach sand, and aerosol at the water's edge along the coast. In addition, this study is unique in that it included measurements by culture-based methods plus the documentation of microbial communities in the SML, bulk water, beach sand, and aerosols during the rare conditions of king tides and a sewage spill.

Materials and methods

Site description and beach characteristics

Sampling was conducted at Darwin Beach (25°43'54.2'N, 80°09'44.1'W), located on the property of the University of Miami Rosenstiel School of Marine, Atmospheric and Earth Science (UM-RMAES) in Miami, Florida. This beach site included a dock fitted with electricity for the vacuum pumps used for air sampling. Samples were collected to the east of the UM-RSMAES pier, which houses a NOAA weather station (station ID 8723214) providing measures of tide, wind direction, wind speed, peak gust, and air temperature. Rainfall data were collected from the WeatherSTEM station on the UM-RSMAES campus. The rainfall data were taken from the previous 24 h from the end of the sampling period. The tidal height was measured using the tidal datum mean lower low water (MLLW). Samples were collected on 15 days ranging between September 15, 2019 and February 23, 2020.

Sample collection

For each of the 15 field efforts, three air samples and seven water samples were collected. Beach sand samples were collected during the last 13 field sampling events. Upon collection, all samples were immediately placed in a cooler with ice packs and were processed at the lab within 1 h of collection.

For the air samples two impactors (SKC BioStage® Single-Stage impactor, 25-mm inlet cone and 103-mm base, designed to hold a 100-mm agar plate, median cut-point of 0.60 μm) and a filter holder (Mesa Labs, M-5017, closed 47 mm diameter) was setup on a tripod for an air sampling period of 3 h (9 am to 12 noon local time for all sampling dates). A three-hour sampling time was chosen to increase the amount of material collected as the levels of microbes in air tend to be low. One of the two impactors was fitted with an agar plate (Marine Agar 2216 (Patrick 1978, Zimbro and Power, 2009), BD Difco™, hereinafter called Marine Agar) for the analysis of GMB. The other impactor was fitted with an mEI agar plate for the analysis of enterococci (US EPA 2014, Method 1600). The filter holder was fitted with a membrane filter (0.45-μm effective pore size mixed cellulose filters, Pall Industries) and this filter was used for subsequent sequencing analysis. Intake for all air collection devices were facing downwards to avoid atmospheric deposition of particles. Sampling devices were set at a height of 0.8 meters above the beach sand within the intertidal zone. Vacuum pump flow rates were set at 30 L/min for the impactors (require a minimum air flow of 28.3 L/min) and 12 L/min for the filter holder (tearing of filters generally occurs at 15 L/min) (Sahwell et al. 2022). Laminar flow rates were maintained at the intakes of both types of air collection devices. Detection limits for the impactors were defined as 0.2 CFU/m^3 air.

The seven water samples were collected at the end of the three-hour air sampling period. Water samples were collected just below the water surface in ankle, knee, and waist deep water using pre-sterilized 1-L Whirlpak™ bags. These samples are called subsurface samples. In addition, at the knee and waist depth water a SML sample was collected by placing a 1.4 mm opening size sieve in the water (following the procedure of Agogué et al. 2004) and then carefully pulling the sieve out of the water and transferring the water captured by the sieve into a pre-sterilized 125 ml Nalgene® bottle. At the waist depth position, two additional samples were collected to better characterize the distribution of bacteria with depth. These additional samples were collected using a Whirlpak™ bag which was opened at mid-depth and at the bottom just above the submerged sediment layer. The sample collection team was careful when entering the water in order to avoid ingestion. After entering the water and collecting the samples, the team members had a change of clothes and showered immediately upon completing field work. Gloves were worn throughout the sample collection process.

Beach sand samples were collected in the supratidal zone (dry beach sand just above the high tide seaweed line), based on a previous study conducted by the researchers which showed that the highest bacteria levels were observed in the supratidal zone (Phillips et al. 2011a). All beach sand samples were collected at the end of the air sampling period by scraping the upper 2.5 cm of beach sand using a pre-sterilized spoon and placing it into a 1 L Whirl-Pak™ bag.

Depiction of the conceptual interactions and of the sampling locations of water, beach sand, and air can be found in Fig. 1 and in the supplement (Fig. S1). As shown in these figures, the aerosol samples were collected in the intertidal zone of the beach, while the beach sand samples were collected in the supratidal zone. Water samples were collected upwind from the aerosol sampling location while the beach sand samples were collected downwind of the aerosol samples. To minimize contamination from human associated microbes, water samples were collected away from where the sampler was standing. SML samples were collected as far away as possible from where the sampler entered the water. Beach sand samples were collected from an undisturbed por-

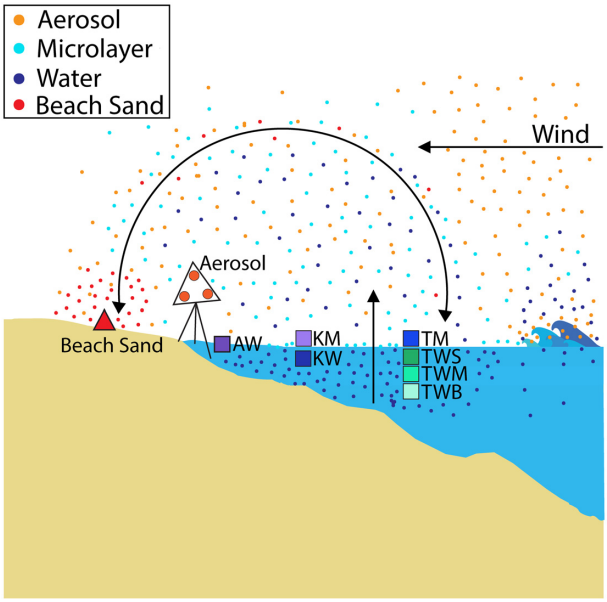

Figure 1. Conceptual diagram showing the interactions between the water, microlayer, aerosol, and beach sand reservoirs. The potential interactions include exchange between the microlayer and the aerosol in the neritic zone, the water supporting the microbial communities in the microlayer, and the beach sand interacting with the water and aerosols. In this study, the winds were originating from the Atlantic Ocean. From this experimental setup, the water samples are upwind of the aerosol sampling location and the beach sand sampling location is downwind from the aerosol samples.

tion of the beach to minimize contamination. The sampling site is rarely frequented by sunbathers given the small shore.

Sample pre-processing

Upon receipt at the laboratory, air, water, and beach sand samples were processed for GMB by culture, enterococci by culture, and for sequencing analysis. All filters prepared for sequencing were placed in a sterile 2 mL tube along with 1 mL of DNA preservative (DNAgard® by Biomatrica®) and then stored in a refrigerator (4°C) until extraction.

The three air samples were processed, by first taking the two sets of agar plates from the impactors, one with Marine Agar (for GMB) and the other with mEI (for enterococci) and separating them into two batches as they each underwent different incubation conditions. The filter from the filter holder was then placed in the DNA preservative for later sequencing analysis.

Similar to the air samples, each of the seven water samples were used to prepare three filters (0.45-μm mixed cellulose filters 47 mm diameter, Pall Industries) using standard vacuum filtration methods. Two filters were used for culture-based analysis (one filter for GMB on Marine Agar and one filter for enterococci on mEI agar) and the third filter was used for sequencing analysis. Prior to filtration, samples were shaken to homogenize. Sample volumes were 1 mL for the analysis of GMB and 100 mL for the analysis of enterococci. The filter sample volumes for sequencing analysis were near the maximum allowable prior to clogging, usually at 600 mL with exact volumes recorded for each sample.

The beach sand samples that were collected were aseptically mixed using a sterile spoon in the Whirl-Pak™ bag to make the sample as uniform as possible. One aliquot of the sand sample was gravimetrically analyzed to obtain the moisture content (dried in an oven set at 110°C for 24 h). A second aliquot was used to extract the microbes from the beach sand according to the procedure described by Boehm et al. (2009). In brief, for this method 10 grams of beach sand were placed in a sterile bottle. One hundred mL of sterile PBS was added, the bottle was shaken for 2 min, and then allowed to settle for another 2 min. The filters used for culture-based analysis received 0.1 mL (Marine Agar) and 10 mL (mEI agar) of this extract. These filters were then placed on their respective agar. The filter used for sequence analysis received 20 mL and was processed for DNA preservation as described above.

Bacterial enumeration

Once the samples were pre-processed, those on Marine Agar (1 air impactor plate, 7 water plates with filter membranes, 1 beach sand plate with filter membrane per sampling event) were incubated at 25 ± 0.5°C for 72 h. At the target incubation periods, colonies that were beige or brightly colored (orange, yellow, dark blue and red) were counted as positive for GMB. Filters on mEI agar plates were incubated at 41 ± 0.5°C for 24 h and blue colonies were counted positive for enterococci. Results for the impactor plates were normalized by the volume of air filtered and were reported in units of colony forming units (CFU) per m^3 of air. Similarly, results for the water samples were calculated in units of CFU per 100 mL. Results for beach sand samples were calculated in CFU per g of dry sand.

16S rRNA gene sequencing

The samples were processed to extract total environmental genomic DNA. To extract the genomic DNA, filters were aseptically transferred to the 'Lysing Matrix E' bead beat tubes (from the FastDNA Spin Kit for Soil, MP Biomedicals). The tube with remaining DNAgard was then centrifuged at 14 000 × *g* for 5 min to concentrate cells that may have been lysed while preserved. No more than 100 μL of the concentrated DNAgard solution was transferred to the bead beat tube with the filters. Samples were then lysed and homogenized with a FastPrep-24 instrument (MP Biomedicals) at an impact speed of 6.0 m/s for 60 sec, and then purified for total environmental genomic DNA from the lysate with the FastDNA Spin Kit protocol (MP Biomedicals, Thermo-Fisher) according to the manufacturer's instructions and stored at −80°C. Details of the extraction process are available in the supplement text. Extracted samples were sent to the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign for Fluidigm amplification and sequencing of the V4 region of 16S rRNA gene. A mastermix for amplification was prepared using the Roche High Fidelity Fast Start Kit and 20x Access Array loading reagent according to Fluidigm protocols (Fluidigm Corp). The final pool was quantitated using Qubit (Life Technologies, Grand Island, NY) and then further quantitated by qPCR on a Bio-Rad CFX Connect Real-Time System (Bio-Rad Laboratories, Inc. CA). Details of the extraction and sequencing process are available in the supplement text.

Bioinformatic analysis was facilitated by the Qiime2 framework. Samples were demultiplexed and primers were trimmed prior to amplicon sequence variant (ASV) table generation using DADA2. Subsequent taxonomic analysis was performed using a Naïve Bayes classifier trained on 99% OTUs from the 515F/806R sequence regions. Data were further filtered for known contaminants based on those identified by Salter et al. (2014).

Statistical analysis

For water, averages for all the seven sub-environments were combined for each sampling day and plotted (Fig. S3 in the supplement). This provided a wholistic picture of the bacteria (GMB and enterococci) on a day-to-day basis. Averages were also computed on an individual sub-environment basis (Tables S1 to S4).

The Shapiro-Wilks test was performed on the cultured GMB and enterococci data to assess the normality of the data and to determine which tests would be used to evaluate correlations. It was determined that the data were not normally distributed, so non-parametric analysis was performed on the data. Kendall's Tau-b was used to evaluate correlations between environmental and bacterial culture data. The significance of the correlation is given by the Kendall's Tau-b correlation coefficient, r with P values less than 0.05 considered significant. The Kruskal–Wallis H test was used to evaluate statistical differences in bacteria levels between different water depths since more than one association could be evaluated consecutively. Mann–Whitney U tests were used to determine the differences in bacteria levels for days that experienced the sewage spill and king tides.

The non-metric multidimensional scaling analysis (nMDS) was conducted on the sequencing data to assess the similarities between the sub-environments. Bray–Curtis was used as the similarity index. To test the significance of differences between the sample types an ANOSIM test was conducted. Similarity percentages (SIMPER) were also used to determine the genera that contributed most to the average dissimilarity of the sample types.

Aerosolization factors

Aerosolization factors (AF) were computed to examine the relative concentration from the water or microlayer to air interaction. In this study the AF was defined on a genera level as the ratio of the % abundance of microbes in air (A) to the % abundance in water (W, Aerosol: Water) or % abundance in SML (M, Aerosol: Microlayer), (Eq. 1) (Michaud et al. 2018). To calculate the AFs, % abundance that contributed <0.01% to the populations in any genera or phyla, were omitted to avoid erroneous calculations. For this analysis, only sampling days were used for which an aerosol sample was available. If an aerosol sample did not have sufficient nucleic acid for sequencing, then the sequences for the other reservoirs were not used. The equation used is provided below.

$$\textit{Aerosolization Factor (AF)} = \frac{\textit{Percent Abundance in aerosol (A)}}{\textit{Percent Abundance in water (W) or microlayer (M)}}$$

Results

Environmental monitoring

Samples were collected over 15 sampling dates (September 15, 2019 to February 23, 2020) in a subtropical environment characterized by low wave energy (tidal height varied from 0.16 to 1.17 m, wind speed varied between 1.6 and 7.8 m/s, with peak gusts reaching 11.1 m/s, and air temperature ranged from 15.8°C to 27.7°C) with winds coming from the Atlantic Ocean. Two of the sampling efforts coincided with king tide (September 29, 2019 and October 27, 2019). Also, a no contact water advisory for the study beach was issued due to a sewage spill from a nearby (2.4 km) wastewater treatment plant during one of the sampling dates (October 13, 2019). Hybrid Single-Particle Lagrangian Integrated Trajectory (HYSPLIT) atmospheric dispersion model was used to confirm air mass sources. More information on environmental data collected and HYSPLIT analysis can be found in the supplement.

Culture-based monitoring of two categories of bacteria: GMB and enterococci

Water samples

There was a statistical difference between the days that did and did not experience king tides for both GMB (Mann-Whitney U, p = 0.012) and enterococci (P = <0.001). The average GMB levels in water on days with king tide was 25 200 ± 15 200 CFU/100 mL (n = 14) in contrast to 16 100 ± 12 600 CFU/100 mL (n = 91) during days without king tide. Similarly, the average enterococci levels were 61 ± 52 CFU/100 mL (n = 14) and 22 ± 29 CFU/100 mL (n = 91) on days with and without king tides.

On the day of the sewage spill, there was a statistically significant increase in enterococci concentrations in water (P = <0.001, 100 ± 41 CFU/100 ml average in water on the day with the spill and 22 CFU/100 mL on average for days without the sewage spill) and no statistical difference in the GMB (P = 0.084). For enterococci in water, the SML samples (knee and waist) were the highest overall on the day of the spill (Fig. 2). The enterococci in the SML at knee depth water measured 177 CFU/100 mL and in the SML at waist depth measured 67 CFU/100 mL. During non-sewage spill conditions these same SML environments measured 13 ± 10 and 12 ± 13 CFU/100 mL, respectively on average. The differences between these SML samples on the spill day and non-spill days were statistically significant (P = 0.009).

When comparing concentrations of GMB and enterococci in water, a significant correlation was observed (Kendall Tau-b, r = 0.282, P = <0.001). High concentrations of enterococci were almost always associated with elevated counts of GMB (Fig. S2). Despite this significant correlation, however, six samples with high concentrations of GMB (> 40 000 CFU/ 100 mL) were low for enterococci. Two of these samples were SML samples.

Beach sand samples

Beach sand samples (collected for 13 of the 15 sampling dates) analyzed for GMB ranged from below detection (1 CFU/g dry sand) (three samples) to 39 700 CFU/g dry sand. GMB concentrations within the dry beach sand was not significantly correlated with the average concentration of GMB in the water for each sampling day (r = −0.013, P = 0.951).

For enterococci, beach sand samples by culture ranged from below detection (1 CFU/g dry sand) (four samples) to 50 CFU/g dry sand. There was no significant correlation observed between enterococci in beach sand and the average concentration of enterococci in water for each sampling day (r = 0.325, P = 0.135). Among the water sub environments, there were no statistically significant correlations with beach sand. Comparison of GMB to enterococci in beach sand showed no significant correlation (r = 0.069, P = 0.754).

Aerosol samples

For GMB by culture, aerosol samples ranged from below detection (0.2 CFU/ m^3 of air) to 65 CFU/ m^3 of air, with approximately a third of the samples below detection limits. A weak but significant correlation was observed between the concentration of culturable GMB in air and culturable GMB in the 105 water samples (r = 0.176, P = 0.014). GMB concentrations in aerosol samples were not significantly correlated with the GMB concentrations in dry beach sand for each sampling day (r = −0.087, P = 0.697). Interestingly, when both SML samples were considered (n = 30), there was a weak but

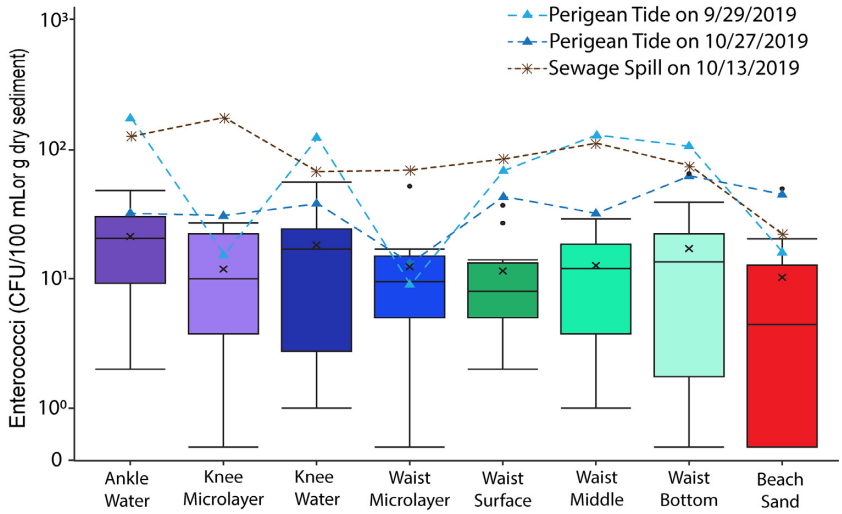

Figure 2. Concentration of enterococci in water and beach sand samples. Lines inside the box plots represent the median, the black '×' in the box represent the arithmetic mean. The boundary of each box closest to zero indicates the 25th percentile and the boundary farthest from zero indicates the 75th percentile. Error bars represent 95% confidence limits. Outliers shown by black dots outside the whiskers. Data collected on the two perigean tide days are represented by triangles and sewage spill data are represented by an '∗'. These data points are not included in the box and whisker plots. All enterococci aerosol samples were below detection limits.

significant correlation between the concentrations of GMB in the SML samples and GMB in aerosol samples ($r = 0.274$, $P = 0.04$). For enterococci, all aerosol samples were below detection (<0.2 CFU/ m^3 of air), so correlations could not be established between enterococci concentrations within other matrices.

Environmental sequencing of 16S rRNA

In total, of the 133 environmental samples submitted for sequencing along with two negative controls, 5245729 sequences were obtained in total and 4914138 sequences were retained after filtering. Sequencing data are available through the SRA database using the following accession number PRJNA838013. Due to low sequence number (the DNA concentration was less than 10 ng/μL or the number of sequencing reads was less than 300), there were a total of 41 samples that were excluded from statistical analyses. The following samples were excluded: 7 ankle water, 4 knee SML, 6 knee water, 3 waist water surface, 8 waist water middle, 5 waist water bottom and 8 aerosol samples. For the aerosol samples that were excluded, one sample corresponded to the day of the sewage spill and one corresponded to one of the king tide sampling days. A table in the supplement shows the characteristics of the samples processed (Table S9).

Within the 4914133 unique sequences, 1754 unique genera were identified. Diversity was analyzed across 92 samples. Diversity varied significantly across sample types (Kruskal–Wallis test, $P = <0.001$) at the genera level. Diversity was lowest in the water samples collected at waist depth in the subsurface and bottom with a mean Shannon Diversity of 1.70–1.71, respectively. Diversity was highest in the beach sand samples with a mean Shannon Diversity index of 4.26 (Fig. 3A). Higher values observed in the beach sand indicated more diversity among species in this sub-environment compared to water and air. Shannon diversity for water samples ranged from 1.70 to 2.72 with the SML samples representing the high end of this range (2.72). Diversity of the aerosol samples was within the range observed for water, at a value of 2.38. Water and aerosol samples overall shared similar diversity indices.

Visualization of the similarity of different environments using non-metric multidimensional scaling (nMDS) analysis (performed on 92 samples for which adequate sequencing data were available) showed clear distinctions among sample types with clusters represented by beach sand, aerosol, and water samples (Fig. 3B). The nMDS ordination is supported by the ANOSIM test which showed significant differences across sample types ($r = 0.31$, $P = 0.0001$). Based on pairwise comparisons, significant differences were found between microbial communities in aerosols and beach sand compared to water and SML samples (Table S10). Statistically significant differences were detected in water sub-environments between the waist subsurface and waist SML sub-environments ($r = 0.12$, $P = 0.01$). Within the water sub-environments, the waist SML showed the largest range with values closest to aerosols. Similarly, the knee SML also showed a large range with values closest to beach sand (Fig. 3B). SIMPER analysis (Tables S11 to S16) reveals the % contribution of the top major genera between the sample types. *Synechococcus* and *Coraliomargarita* were the top genera between aerosol and water samples, aerosol and microlayer samples, and water and microlayer samples, with higher levels found in the water and microlayer samples. Beach sand samples differed in *Synechococcus* and *Nocardioides* abundances when compared to microlayer and water samples. Finally, between the aerosol and beach sand samples the abundances of *Nocardioides* and *Lysobacter* also differed (Fig. 4).

Water

The dominant genera (Fig. 4) in the SML and bulk water samples included *Synechococcus* (average of 20% and 34%, respectively) and *Coraliomargarita* (average of 15% and 7%). Similarly, in the bulk water samples, *Synechococcus* was detected in highest abundance. When comparing the SML samples to the bulk water samples, *Staphylococcus* and *Vibrio* were more prominent in the SML (average of 0.25% and 0.58% respectively) than the bulk water (average of 0.14% and 0.40% respectively). *SAR86 clade* (common heterotrophs in the surface ocean, Hoarfrost et al. 2020), *and OM43 clade* (common methylotrophs in marine environments, Jimenez-Infante et al. 2016) were also noticed in the bulk water and SML (average val-

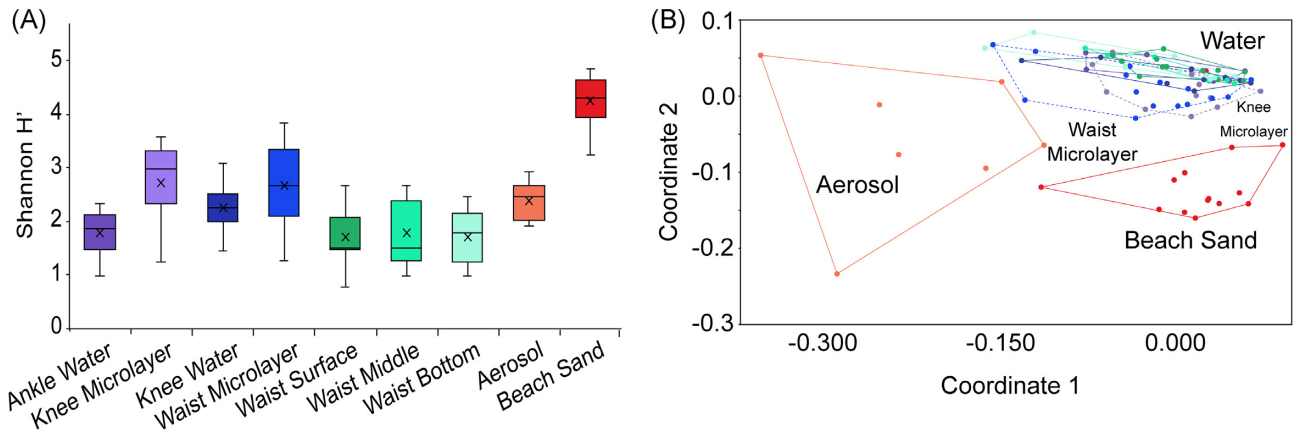

Figure 3. Shannon diversity index of sub-environments at the genus level (panel **A**). nMDS plot of samples across sub environments. The knee SML is represented by the light purple dashed line and the waist SML is represented by the royal blue dashed line (panel **B**).

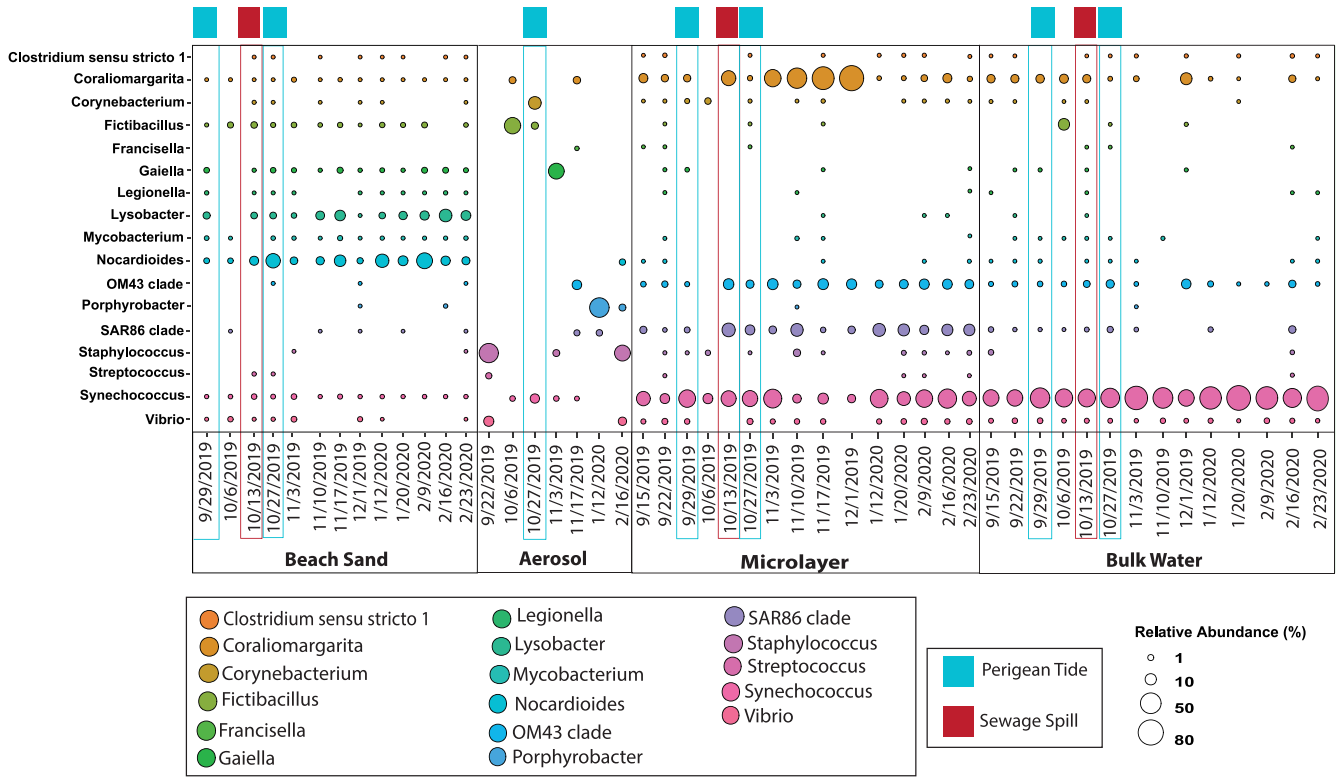

Figure 4. Bubble plot depicting the most abundant genera which include potential pathogens. Dates that experienced perigean tide are outlined in blue boxes and the day of the sewage spill is outlined in red boxes. The microlayer (knee and waist) and bulk water samples (ankle, knee, waist surface, waist middle, waist bottom) were grouped together based upon the MDS plot. *Vibrio, Synechococcus, and Coraliomagarita* are dominant in the water samples, while *Gaiella, Legionella, Lysobacter, Mycobacterium,* and *Nocordioides* are sourced from the beach sands. In the aerosol samples *Fictibacillus, Corynebacterium,* and *Gaiella* are found in high abundance.

ues ranged from 2.3% to 5.8%). On days that experienced king tide, there was a noticeable increase of *Vibrio* (increase of 60% and 90%) in both SML and bulk water samples and of *Corynebacterium* (77% increase) in SML. *Vibrio* and *Corynebacterium* are genera that include known pathogenic bacteria. *Francisella* in the SML increased by 576% on the days that experienced king tide. During the sewage spill, *Mycobacterium* increased by 38% in SML and by 306% in bulk water. *Legionella* was observed to increase in the bulk water samples by 60%. *Francisella* increased in the bulk water samples by 485%. *Enterococcus* was not detected by V4 gene sequencing for microlayer and water samples. Direct comparisons with culturable data could not be completed.

Beach sand

Nocardioides (average of 8%) and *Lysobacter* (average of 4%) were the dominant genera (Fig. 4). Given the rare events that occurred during the sampling effort, there were increases in the proportion of some genera that included potential pathogenic bacteria. During king tide, the proportions of *Corynebacterium, Mycobacterium, Legionella, and Streptococcus* in beach sand increased between 34%

and 163%. Also during king tide days, *Rickettsia* (detected solely in beach sand and bulk water samples) experienced an increase of 231%. During the sewage spill, the proportion of *Corynebacterium* and *Streptococcus* increased by 1800% and 2900%, respectively. *Clostridium sensu stricto 1* also exhibited an increase of 125%. *Enterococcus* was only detected in one of the 13 sequenced sand samples.

Aerosol

Dominant genera (Fig. 4) included *Staphylococcus* (average of 10%), *Porphyrobacter* (average of 6%), *Fictibacillus* (average of 4%), and *Gaiella* (average of 4%). These genera dominate uniquely in the aerosol samples (Fig. 4 and Fig. S3). Similarly, the dominant genera in the SML samples were not dominant in aerosol samples. For example, *Synechococcus* was the most abundant in the SML samples while it was detected at less than 1% abundance in the aerosol samples. For water SML and aerosols, there was common detection of the genera of potentially pathogenic bacteria ((*Corynebacterium* (average of 0.08% and 2%, respectively) and *Vibrio* (average of 0.58% and 1.6% respectively)) at lower proportions. *Enterococcus* was not detected in the aerosol samples and therefore a direct comparison of the culturable data could not be conducted.

Aerosolization factor

Using the aerosolization factor (AF), results show that more than 50% of the genera were concentrated in the aerosol phase as shown by most data points within the top-right quadrants of Fig. 5. The bottom left quadrant indicates the genera that were primarily found in the non-aerosol matrix. A linear pattern is noticeable for the comparison of the water ratios. It can be noted that the genera that include potentially pathogenic species are found in the upper right quadrant. These include those in high abundance as described above (*Corynebacterium*, *Vibrio,* and *Staphylococcus*) and those found in lower abundance (*Streptococcus* and *Francisella*), all of which had high AF values. This can possibly be attributed to the interaction of the bulk water, SML, and air.

Discussion

Based on data from the present study, the SML GMB concentrations were significantly correlated with aerosol GMB concentrations, suggesting interactions between these two environments. This is consistent with the conceptual model of O'Mullan et al. (2017) in which sea spray aerosol (SSA) is generated in the nearshore environment and transported onshore. Dueker et al. (2017) found that wind increases the microbial aerosol number concentrations in the near shore environment by onshore transport. In this study, mean wind speed exceeded the 4 m/s threshold identified by Monahan et al. (1983) 60% of the time suggesting that a majority of the time conditions were conducive towards aerosolization. At higher wind speeds, the portion of large/coarse aerosols increases, which can also lead to a greater connection between the water and air samples. Lower wind speeds disturb the water less and decreases the creation of white caps and thus bubble bursting. There is also lower intensity of shoreline interaction because of lower wind speeds which decrease the production of large/coarse aerosols (Montero et al. 2016). Thus the weak but significant correlation between the GMB in the air and the GMB in the water, is consistent with conceptual models that support the transfer of water to the aerosols phase.

Microbial community differentiation

Based on 16S rRNA sequencing analysis, we identified distinct communities associated with the water, air, and beach sand. Even though they are distinct communities as suggested by the ANOSIM test, there are numerous OTUs that were shared within the environments. Of the 1152 genera identified in the SML, 102 (9%) were shared with the aerosols, 865 (75%) with the bulk water, and 694 (60%) with the beach sand, suggesting interaction between environments. This is consistent with the conceptual model of aerosols being generated by breaking waves and bubble bursting (Dueker et al. 2011, Dueker et al. 2011). Cho and Hwang (2011) found that bacteria in aerosol samples were comparable with those of a marine water origin. This study has confirmed that finding. Other studies (Urbano et al. 2011, Xia et al. 2015, Dueker et al. 2017) found a mix of bacteria from marine and terrestrial origin in aerosol samples. Further evidence of a combination of marine and terrestrial bacteria in aerosol samples can be seen in this study. Diversity among the three environments was in the 2.00 to 4.30 range with Shannon indices of 2.38 for air, 2.09 for water, and 4.26 for beach sand.

From this study, it was found that there were certain genera in the aerosol samples that were found in higher relative abundance than in microlayer, water, or beach sand samples. The differences in genera abundances could represent either a mixing of multiple microbial sources, differential aerosolization of microbial taxa, or a combination of these processes. High AF values were also found to be associated with potentially harmful microbes since the microbes tend to belong to highly aerosolized classes (Michaud et al. 2018). For example, *Staphylococcus*, which includes species of bacteria that cause diseases, exhibited an AF microlayer value of 143 and an AF water value of 373. Consistent with its high aerosolization factors, *Staphylococcus* was the most abundant genera in the aerosol samples (average value of 10%), while it was found to be less than 1% average of the bacteria in the water, SML, and beach sand samples. Additional potentially pathogenic genera found in the aerosols included *Corynebacterium* (AF = 2900 and 15.9), *Streptococcus* (AF = 31.7 and 37.8), and *Vibrio* (AF = 5.59 and 2.54) with AF values representing water and SML, respectively. The AF values for these potential pathogens were all above 2.00, which indicate that they can be readily aerosolized.

While experimental studies suggest aerosolized microbes are likely sourced from nearby surface waters (Michaud et al. 2018, Schiffer et al. 2018, Shaharom et al. 2018, Malfatti et al. 2019, Robinson et al. 2019), this aerosolized community is likely to be shaped by a number of factors. To begin, nearshore waters are heavily influenced by shedding of microbes from coastal beach sands (Whitman et al. 2014, Korajkic et al. 2019), influenced by environmental conditions such as tidal height, UV radiation, and others (Heaney et al. 2014), potential terrestrial sources (soil, plants, and others) (Nayak et al. 2019), and by potential human and animal sources (Elmir et al. 2007, 2009, Wright et al. 2009). The microbial communities of the nearshore waters vary both with depth and distance from shore (as described below), with some of the most significant differences associated with the SML which may reflect the influence of UV irradiation coupled with intermittent inputs such as those associated with sewage spills and interaction of beach sands during king tides and possible impacts from long-range transport. Also, the positioning of where the samples were collected may have also influenced the type of microbes detected in each reservoir as the water samples were upwind of the aerosol sampling location and beach sand samples were downwind from the aerosol sampling location.

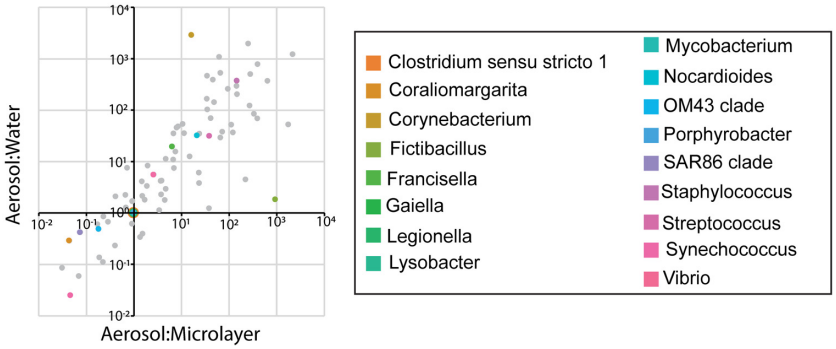

Figure 5. Aerosolization of bacteria across sampling efforts (n = 43). The ratio of the % abundance in aerosols to the % abundance in water is plotted against the ratio of the % abundance in aerosols to % abundance in the SML. The lower left quadrant represents the genera that are not preferentially aerosolized. *Coraliomargarita* and *Synechococcus*are are located in this area. The upper right quadrant represents genera that are preferentially aerosolized including genera that contain potentially pathogenic bacteria (*Staphylococcus*, *Streptococcus*, *Vibrio*, *Corynebacterium*, and *Francisella*). Located in the cross section of the axis are those genus that exhibited a '0' value for detection in aerosol samples (*Mycobacterium, Lysobacter, Legionella, and Clostridium sensu stricto1*). Gray dots in the figure illustrate additional genera not listed in the legend of the figure.

Sewage spill

Sewage is predominantly freshwater and is therefore more buoyant than seawater. As a result, sewage spilled near the top of the water's surface was likely preferentially transported along the surface where the SML samples were collected. On the day of the freshwater sewage spill wind speed was relatively high (~7 m/s), and according to HYSPLIT the wind direction was coming from the Atlantic. Tide was also higher during the day with the sewage spill compared to other days. It is likely that the wind was pushing the more buoyant freshwater sewage towards the shore, resulting in the increased levels of enterococci in the SML during the day of the sewage spill.

On the day of the freshwater sewage spill, the SML samples (knee and waist) exhibited elevated levels of GMB and enterococci. This is contrasted by low levels detected in the SML samples throughout the remainder of the samples. Low levels in this sub-environment are expected, due to solar inactivation of bacteria (Reed 1997, Yukselen et al. 2003, Enns et al. 2012, Maraccini et al. 2016, Nelson et al. 2018). Since sewage is a well-documented source of enterococci (Roca et al. 2019, Ahmed et al. 2020), elevated levels in the water during the spill are expected. The actual enterococci levels observed in the water ranged from 65 to 177 CFU/100 mL, which is still low but impacted compared to times without sewage spills. The proportions of some genera of bacteria that include potential pathogens increased in the water samples on the day of the sewage spill. These genera included those found in high abundance in the bulk water, *Mycobacterium* (306% increase) and *Legionella* (60% increase), and those found in lower abundance, *Francisella* (485% increase), and *Vibrio* (3.5% increase). The fact that the proportions increased within the water SML are particularly relevant, given the proximity of the air and water. Even though a direct relationship between the levels of bacteria in aerosol and beach sand was not observed, environmental factors such as tides and wind were shared between the two environments and potentially increased the transfer of bacteria from beach sand to water and ultimately the aerosolization of bacteria from the water's surface. Beach sand samples exhibited the highest increase in potential pathogenic genera. The proportion of *Streptococcus* increased by 2900%, *Corynebacterium* increased by 1800% and finally the proportion of the lower abundance *Clostridium sensu stricto1* increased by 125%. Although the culturable enterococci levels and potential pathogenic genera increased significantly, the level of enterococci in the impacted beach water was relatively low (on the order of 100 CFU/100 mL) yet higher than during non-sewage impacted periods (about 22 CFU /100 mL). If the sewage spill impacts were greater, it is possible that the pathogenic pathogens could be further enriched in the aerosols during more severe sewage spills. Interestingly there was no overlap in the potential pathogenic genera between water and beach sand during the sewage spill, suggesting that these two reservoirs were not exchanging bacteria during the sewage spill.

Interaction of beach sand during king tide

Subsurface water samples at ankle depth and bottom water samples at waist depth experienced higher levels of GMB and enterococci. These two sub-environments are in close proximity to sediment. Wave action may release bacteria from sediment and introduce it to the shallow water column at the ankle depth (Wright et al. 2011). There was a statistical difference in both the GMB (Mann–Whitney U, $P = 0.012$) and enterococci ($p = <0.001$) in water samples between the days that did and did not experience king tides, likely caused by the release of bacteria from sand. During king tides, water levels were higher and inundated larger portions of the beach sand. Beach sand is a known source of enterococci (Shah et al. 2011, Wright et al. 2011, Phillips et al. 2011b, Piggot et al. 2012) with the release from the sand influenced by tidal cycles (Boehm and Weisberg 2005, Feng et al. 2013, Feng et al. 2016, Aslan et al. 2018), wind (Lewis et al. 2013, Zimmer-Faust et al. 2018), and waves (Phillips et al. 2014).

In a similar fashion bottom samples at waist depth may have experienced higher levels of bacteria possibly because of its proximity to the submerged sediment floor. Resuspension of sediments from the bed floor could contribute to the higher levels of bacteria in the bottom waist samples (Wyness et al. 2019). High winds resulting in greater sediment resuspension can increase the turbidity, which can prolong the survival of fecal indicator bacteria through lowered solar inactivation (Shibata et al. 2010, Aragonés et al. 2016, Laureano-Rosario et al. 2019). These same high winds can promote the aerosolization of bacteria from the water column through the increase in wave conditions which promote the formation of aerosols. During king tide events, there was an increase in the abundance of potential pathogenic genera, specifically in the beach sand samples. Out of the 21 genera of concern, six experienced an increase in the sediment, three increased in the SML, and two in the bulk water samples. Of the 6 genera identified, the most concerning increase was *Streptococ-*

cus (119% increase) as well as *Legionella* (163%). The other genera included *Rickettsia* (231% increase), *Mycobacterium* (120% increase), *Clostridium sensu stricto 1* (80% increase) and *Corynebacterium* (34% increase). For the two genera shared between the bulk water and SML samples, *Francisella* showed a 191% increase in water and 546% increase in SML, and *Vibrio* showed a 60% increase in SML samples, which further supports the interaction between the two environments. *Corynebacterium* was shared between the beach sand and SML samples on the king tide days. Since the beach sand at the shoreline's edge is in contact with the SML, there may be some interaction between the two environments.

Overall results show that levels of bacteria in the SML ranged from 1100 CFU/100 mL to 53 200 CFU/100 mL for culturable GMB and from 1 CFU/100 mL to 177 CFU/100 mL for enterococci. In aerosols levels ranged from below detection (0.2 CFU/ m^3 of air) to 65 CFU/ m^3 of air for GMB and below detection limits (< 0.2 CFU/ m^3 of air) for enterococci. Despite the relatively low levels of aerosolized bacteria, evidence suggests that aerosols were influenced by water quality as levels of GMB in the aerosols correlated with levels in SML water. Further evidence is provided by microbial community analyses, with common genera of bacteria shared between the aerosol, water, and beach sand matrices. Given the evidence connecting water and aerosol microbial quality as observed in the current study and in other studies, there is the possibility of aerosolization of pathogens contained in sewage. This was further supported by the data collected during the sewage spill which showed elevated levels of bacteria and enterococci in the SML samples. During rare events, there were several potentially pathogenic bacteria that experienced an increase. *Streptococcus* (2900% increase) and *Corynebacterium* (1800% increase) were observed to increase in abundance within the beach sand during the spill. On the days of king tide, beach sand exhibited an increase in abundance in *Legionella* (163% increase), while *Vibrio* increased in the water SML (57% increase).

Overall genera that contain potentially pathogenic bacteria were observed to increase in water and beach sand during rare events. The increases to the SML were likely driven by buoyancy effects in the case of freshwater sewage releases and proximity of the SML to beach sand at the shoreline. Given the high AF factors for some of these genera, research is needed to prepare for possible future disease outbreaks of respirable pathogens that can be found in sewage. An emphasis should be placed on microbes characterized by high AFs.

Author contributions

Afeefa A. Abdool-Ghany (Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft), James S. Klaus (Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing), Luis E. Sosa Villegas (Formal analysis, Investigation, Methodology), Trent D'Alessio (Formal analysis, Investigation, Methodology), Maribeth L. Gidley (Conceptualization, Formal analysis, Methodology, Resources, Supervision, Writing – review & editing), Christopher D. Sinigalliano (Conceptualization, Formal analysis, Methodology, Resources, Supervision, Writing – review & editing), Cassandra Gaston (Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing), and Helena M. Solo-Gabriele (Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing).

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Supplementary data

Supplementary data are available at *FEMSEC* online.

Conflicts of interest statement. None declared.

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