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Antibiotic-Resistant *Escherichia coli* and *Enterococcus*Spp. in Sand and Water at Tampa Bay Beaches

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by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
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ABSTRACT

As antibiotic resistance in the environment continues to rise there is an increased concern that infections may become harder to treat as bacteria acquire genes for multidrug resistance. Recreational beach waters in the Tampa Bay area are routinely monitored by the Florida Department of Environmental Protection for the presence of fecal indicator bacteria (FIB) such as *Escherichia coli* and enterococci. Exceedances of beach action values (BAV) 235 CFU/100 mL (*E. coli*) and 70 CFU/100 mL (enterococci) indicate the presence of fecal contamination which is associated with an increased risk of disease for beachgoers. Antibiotic-resistant *E.* coli and *Enterococcus* spp. have been studied in recreational beach waters but have not been as thoroughly researched in sand. This study investigated the frequency and concentration of antibiotic-resistant *E. coli* and *Enterococcus* spp. in water and sand from three recreational bay beaches in the Tampa, Florida area that have historically elevated BAV.

The frequency and concentration of ampicillin-resistant *E. coli* and erythromycin-resistant *Enterococcus* spp. in water and sand (swash zone, and 2.5 meters away from the swash zone) at these beaches was compared to the frequency of multi-drug resistant *E. coli* and *Enterococcus* spp.. Each beach was sampled three times from March 2021 to June 2021. *E. coli* was isolated on Media with and without ampicillin (16 µg/mL) and enterococci with and without erythromycin (4 µg/mL) selecting for intermediate resistance. Thirty percent of all *E. coli* isolates showed intermediate phenotypic resistance to ampicillin and were significantly higher in both

concentration ($P < 0.05$) and proportion ($P < 0.05$) 2.5 meters away from the swash zone. Of the isolates that expressed ampicillin resistance, 97% were confirmed to species and then subjected to susceptibility testing to seven antibiotics. Twenty-one percent of ampicillin-resistant *E. coli* isolates showed multiple-antibiotic resistance (i.e. resistance to three or more antibiotics). Multidrug-resistant *E. coli* were observed to be significantly higher 2.5 meters away from the swash zone ($P < 0.05$).

Forty-one percent of all enterococci isolates showed intermediate phenotypic resistance to erythromycin and were significantly higher in both concentration ($P < 0.1$) and proportion ($P < 0.05$) 2.5 meters away from the swash zone ($P < 0.05$). Of the isolates that expressed erythromycin resistance, 83% of were confirmed to the genus *Enterococcus* and subjected to susceptibility testing to six antibiotics. Fifty-four percent of *Enterococcus* spp. isolates showed multidrug resistant patterns. These results express that sand does serve as a source for single and multidrug-resistant *E. coli* and *Enterococcus* spp. that could potentially infect beach recreators.

CHAPTER ONE:

ANTIBIOTIC-RESISTANT *ESCHERICHIA COLI* AND *ENTEROCOCCUS* SPP. IN SAND AND WATER AT TAMPA BAY BEACHES

Antibiotic-Resistant Bacteria

One of the largest human health risks the world is currently facing is the growing prevalence of antibiotic-resistant bacterial pathogens [1]. Currently, 2.8 million people in the U.S. acquire infections from antibiotic-resistant bacteria (ARB) per year. Of those, at least 35,000 die each year as a result and these numbers continue to rise [2]. The increase in antibiotic resistance worldwide has been mainly attributed to antibiotic overuse in the health sector, but this same issue is also prevalent in agriculture, animal husbandry, and aquaculture [3–6]. Human exposure to ARB occurs in the clinical setting, but also through environmental routes including contaminated food, water, and sand [7,8]. The occurrence of ARB in the environment can be attributed to the poor absorbance of antibiotics in the gut of animals, 30-90% of antibiotics are excreted back into the environment including water bodies [3,4,9].

The responses that bacteria have to antibiotics can be classified into three clinically significant categories: susceptible (growth highly inhibited by clinical dosage of antibiotic), intermediate resistance (growth is inhibited, but to a lesser extent than susceptible bacteria), and full resistance (not inhibited by clinical dosage of antibiotic) [10,11]. Both intermediate and fully-resistant bacteria pose a clinical threat, and

intermediate-resistant variants frequently have the ability to rapidly evolve towards a higher resistance level [10]. Pressure on bacteria applied by the presence of antibiotics favors the selection of antibiotic-resistant variants, which can be initiated by the use and misuse of antibiotics [12]. Antibiotic-resistant phenotypes arise through genetic mutations or acquisition of antibiotic resistance genes via horizontal gene transfer by mobile genetic elements (MGE) (i.e., transposons and integrons) [12,13]. A primary concern is ARB transferring their antibiotic resistance genes to pathogenic bacteria that are susceptible to that antibiotic, or pathogens that already express resistance to other antibiotics, which can lead to hard-to-treat multi-drug resistant (MDR) strains which require the use of last resort antibiotics [14].

Escherichia coli and *Enterococcus* spp. are both commensal bacteria that are part of the normal gut flora but can also be opportunistic pathogens and considered fecal indicator bacteria (FIB) [15]. *E. coli* and *Enterococcus* spp. are a common cause of diseases such as urinary tract infections, soft tissue infections, bacteremia, endocarditis, and meningitis [14,16–21]. In the 2019 CDC Antibiotic-Resistant Threats Report, ESBL-producing Enterobacteriaceae, which includes both *E. coli* and vancomycin-resistant *Enterococcus* spp. were designated as serious threat and contributed to 197,400 and 54,500 hospital cases in 2017 respectively [2,22].

E. coli are highly studied gram-negative bacteria that are frequently isolated in clinical specimens and can carry virulence factors, making them pathogenic [16,19]. Pathogenic categories of *E. coli* are associated with a high morbidity and mortality rate and include intestinal strains that cause gastrointestinal (GI) disease and extraintestinal strains which are the cause of many infections outside of the GI tract (i.e. pneumonia and

sepsis) [23].These infections are normally treated with various types of antibiotics like penicillins and β -lactam/β- lactamase inhibitor but the increase in antibiotic resistance has made treatment more difficult [19]. *E. coli* have relatively high rates of acquired resistance (not innate), for example they carry beta-lactamase producing genes which mediate resistance to beta-lactams which include antibiotics with clinical importance like ampicillin [24,25].

Enterococcus spp. belong to a gram-positive commensal genus which include 23 species, of these 12 are considered to be opportunistic pathogens [26,27]. Two of these species are considered to be the leading causes of nosocomial (hospital acquired) infections, *Enterococcus faecalis, and Enterococcus faecium* [26–28]. *Enterococcus* spp. have a high level of intrinsic resistance to many antibiotics, including aminoglycosides, beta-lactams, and lincosamides [29]. *Enterococcus* spp. can also acquire antibiotic resistance genes to vancomycin, erythromycin, and fluroquinolone through MGEs [30,31]. Because of this, measuring acquired erythromycin resistance is of interest due to its clinical relevance in treating *Enterococcus* spp. infections [14,32]. Erythromycin is classified as a macrolide, which is the most commonly prescribed antibiotics worldwide [33,34].

Antibiotic-Resistant Bacteria in Recreational Beach Water

As awareness of ARB in the environment continues to increase, examining human exposure routes as well as reservoirs of ARB has gained interest [35,36]. Recreational beach waters have been shown to harbor FIB such as *E. coli* and *Enterococcus* spp., which can signify the presence of sewage contamination [37]. Pathogens associated with sewage contamination cause gastrointestinal illness through primary contact (e.g. putting one's

head under water or ingestion) [38,39]. Pathogens that survive in beach water create a risk for individuals wading or swimming in the water body, especially children and elderly because of their developing or weakened immune system [38–40].

Antibiotic-resistant FIB are not only a concern because they are opportunistic pathogens, but also because there is a potential for horizontal gene transfer of their resistance genes to other harmful pathogens (*Salmonella* spp., *Legionella* spp., and *Pseudomonas* spp.) that can be found in sewage-contaminated waters [41]. Because of the increased prevalence of ARB in the environment, research on antibiotic resistance have broadened to include water bodies, sands, and sediments that may act as reservoirs or points of human exposure. In the environment, acquisition of multiple resistance genes through horizontal gene transfers and spontaneous mutations has been identified [42]. Antibiotics have been detected in surface waters, which may exert selective pressures on the bacteria and result in an increased proportion of ARB and antibiotic resistance genes (ARG) [9,43–45]. This is particularly concerning in recreational waters where primary contact frequently occurs. The ability for *E. coli* and *Enterococcus* spp. to acquire ARG make them both indicators of sewage contamination and ARG presence[43,46,47]. While there are many studies of antibiotic-resistant bacteria in water, there is a lack of literature exploring antibiotic-resistant *E. coli* and *Enterococcus* spp. in sand.

Antibiotic-resistance Bacteria in Recreational Beach Sand

Sand is a less explored habitat of ARB and ARG compared to water, despite the fact that beach recreators generally interact with sand even if they choose to not wade in the water [48]. High concentrations of *E. coli* and *Enterococcus* spp. can inhabit the sand

environment and may be autochthonous in sand [14,48,49]. Sand can be an advantageous environment for bacteria because of its physical protection from ultraviolet radiation as well as its capability for retaining and obtaining nutrients through wrack and biofilms that wash up onto the sand-water interface [48,50,51]. The literature shows that not only can beach sands can be contaminated with *E. coli* and *Enterococcus* spp. but that these sands can also support antibiotic-resistant variants of these bacteria [39,48,52]. It has been shown in previous studies that *E. coli* and *Enterococcus* spp. concentrations in sand can be higher than that of the water column, which could lead to an increased risk of infection as well as drug resistant infections [39,53,54]. The pathogenic variants of *E. coli* and *Enterococcus* spp. that persist in the sand environment can cause illness through accidental ingestion of sand on recreational beaches, or indirectly by reintroduction into the waterbody in the swash zone (the portion of the beach where the waves interact and mix with the sand) [14,48,50,55].

The U.S. Environmental Protection Agency (EPA) does not have any standards for allowable levels of fecal microorganisms in sand; yet we know that beach goers can become ill following contact with sand [56–59] . The extent to which sand could serve as an exposure route to antibiotic-resistant pathogens is not well-understood, but a first step is to explore the levels of antibiotic-resistant, fecal bacteria in sand. This research determined the concentration and frequency of single resistant and multidrug-resistant *E. coli* and *Enterococcus* spp. in the water and sand matrix of three Florida recreational bay beaches. The predictions I have made for this research are (1) that concentrations of single-drug resistant *E. coli* and *Enterococcus* spp. will be higher in sand than in water,

and (2) I expect a higher frequency of multi-drug resistant variants in sand compared to water.

METHODS

Site Description

Three sample sites were chosen in the upper Tampa Bay that had high population densities in the surrounding watersheds, and historical enterococci data available from the Florida Healthy Beaches Program (https://www.floridahealth.gov/environmental-health/beach-water-quality/index.html). Environmental parameters which could correlate with *E. coli* and enterococci levels (i.e. temperature, salinity, turbidity, and pH) were measured at each site. Three sampling events at each beach occurred at mid-tide during an ebb current from March to June 2021. Courtney Campbell Beach (CC) (27°57'38.0"N 82°41'58.1"W) is located at the end of the Courtney Campbell bridge on the edge of Clearwater. This beach is approximately 20 meters from the highway, situated between two major highway bridges, the Courtney Campbell Causeway, and the Bayside Bridge. Ben T. Davis Beach (BT) (27°58'04.3"N 82°34'23.7"W) is located on the eastern most end of the Courtney Campbell Causeway and is about 50 meters from the highway in a highly urbanized area with many restaurants and hotels surrounding this sample site. The third beach is the southernmost beach, Cypress Point Park (CP) (27°57'0.3"N 82°34'45.6"W) and is the furthest removed from the highways of the three beaches. This sampling location has pavilions, a playground, walking trails and extensive vegetation separating the beach from the urban areas that surround it.

Water Sampling

Environmental water samples were collected from three Tampa Bay beaches (Figure 1.). At each beach, three 1 L grab samples of water were collected at approximately 30 cm (ankle) depth using a sterilized 1 L polypropylene container in 30 m increments. The three grab samples were combined into a 3 L composite sample as a representation of the entire beach. The water samples (W) were designated as CC-W (Courtney Campbell), BT-W (Ben T. Davis), and CP-W (Cypress Point Park). Water samples were immediately placed on ice, transported to the laboratory, and processed within 1-2 hours after concentration by membrane filtration using USEPA methods (56).

Sand Sampling

Sand samples were collected at two subsites per beach, the swash zone sand and 2.5 meters from the swash zone in foreshore sand. These subsites were designated as follows CC-SZ (swash zone), CC-FS (foreshore), BT-SZ, BT-FS, CP-SZ, and CP-FS. At each subsite, 150 g samples of sand were collected using a sterilized 500 mL polypropylene container. These samples were taken from the upper two inches of the sand surface at three transects in 30 m intervals along the beach. Once these samples returned to the lab, they were homogenized into a 450 g composite sample using a sterilized hand mixer. The sand samples were processed within 1-2 hours after collection through standard membrane filtration using USEPA methods [60,61].

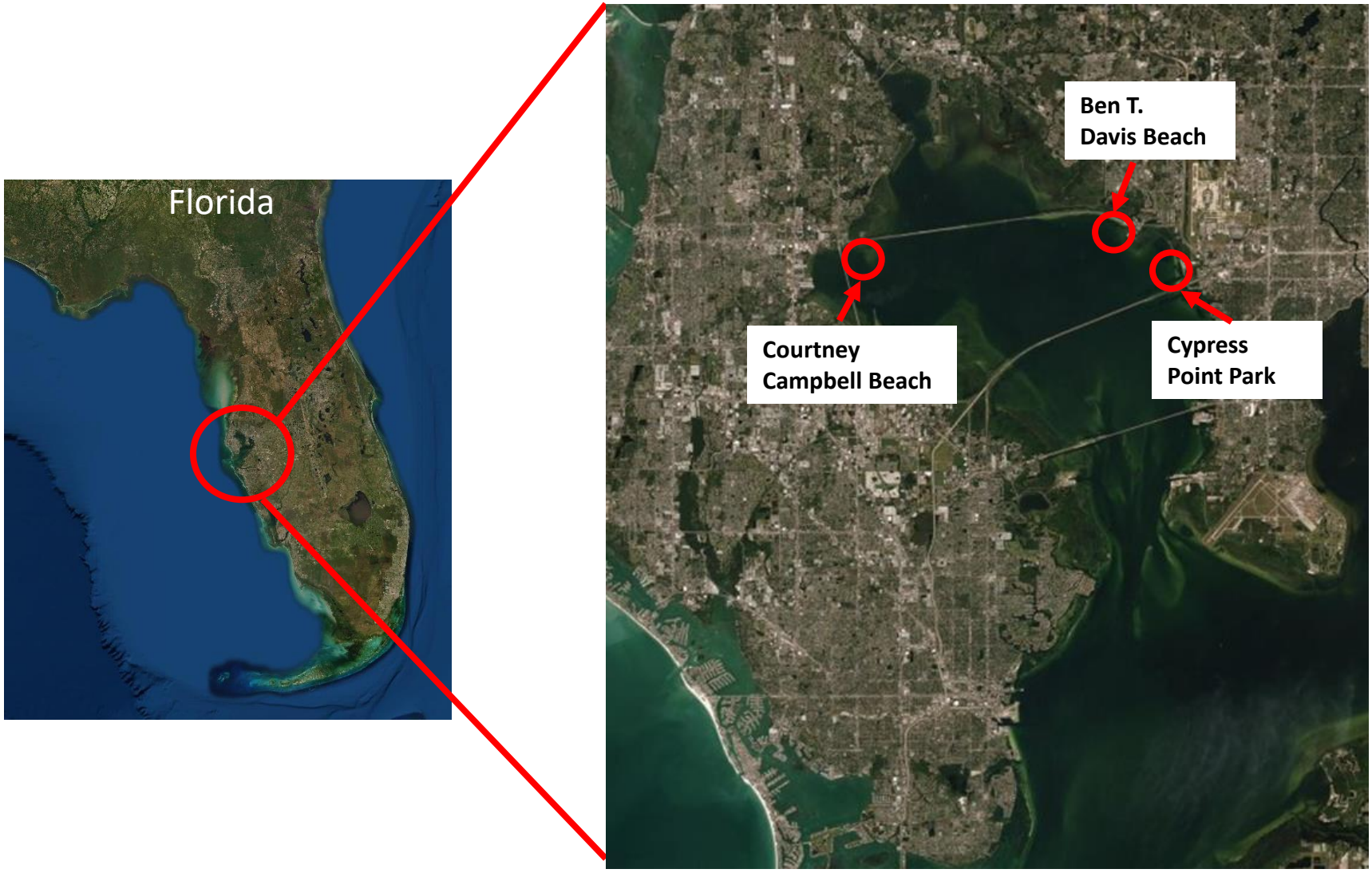

Figure 1. Sampling sites in the Tampa Bay area. Satellite image acquired from Google Maps (Imagery ©2021 Terramatreics, map data © 2021 Google).

Enumeration of Total and Antibiotic-Resistant *E. coli* and Enterococci From Environmental Water

Each of the water samples was processed using the standard membrane filtration method (USEPA method 1603 for *E. coli* and USEPA method 1600 for enterococci*)* [60,61]. in volumes of 1 mL, 10 mL, and 100 mL using 0.45 µM (47 mm diameter) nitrocellulose membranes (ThermoFisher Scientific, Waltham, MA, USA) for total *E. coli* and enterococci. Volumes of 100 mL and 500 mL (or to refusal) were filtered in the same manner for intermediate antibiotic-resistant *E. coli* and enterococci. Membranes were placed on mTEC amended with and without ampicillin (16 µg/mL) and mEI amended with and without erythromycin (4 µg/mL) plates for the isolation of antibiotic-resistant *E. coli* and enterococci, respectively. These antibiotic concentrations were selected for intermediate resistance [11]. The decision to select for intermediate, rather than full resistance, was made to ensure that antibiotic-resistant bacteria were detected. The mTEC agar plates were incubated at 35°C for 2 hours followed by incubation at 44.5° C for 18-20 hours in a water bath, mEI agar plates were incubated at 41°C for 22 hours [60,61]. After the incubation, colonies were counted, and concentrations expressed per 100 mL of water.

Enumeration of Total and Antibiotic-Resistant *E. coli* and Enterococci From Environmental Sand

Bacteria were isolated from sand using the slurry method [62,63]. A one to ten dilution (400 g/3600 mL) of sand to phosphate buffer solution (PBS) was made and then agitated for three minutes followed by a settling time of 30 seconds [64]. The supernatant from this slurry was decanted and processed using the standard membrane filtration method

(USEPA method 1603 for *E. coli* and USEPA method 1600 for enterococci*)* [60,61]. For *E. coli* and enterococci, volumes of 1 mL, 10 mL, and 100 mL for each sand sample was passed through 0.45 µM (47 mm diameter) nitrocellulose membranes (ThermoFisher Scientific, Waltham, MA, USA) which is an equivalent of 0.1 g, 1.0 g, and 10 g of sand respectively. For antibiotic-resistant *E. coli* and enterococci, volumes of 100 mL and 500 mL (or to refusal) was filtered in the same manner which is an equivalent of 10 g and 50 g of sand respectively. Membranes were placed on mTEC amended with and without ampicillin (16 µg/mL) and mEI amended with and without erythromycin (4 µg/mL) plates for the isolation of *E. coli* and enterococci, respectively. The concentration of antibiotics that the plates were amended with selected for intermediate resistance [11]. The mTEC agar plates were incubated at 35°C for 2 hours, followed by incubation at 44.5° C for 18-20 hours in a water bath, mEI agar plates were incubated at 41°C for 22 hours [60,61]. After the incubation, colonies were enumerated and expressed per 100 g of sand.

Isolation and Confirmation of Antibiotic-Resistant Strains of *E. coli* and *Enterococcus* spp.

Up to 15 colonies each from water, swash zone sand and sand 2.5 away from the swash zone were collected and streaked for isolation onto mTEC and mEI for *E. coli* and enterococci respectively. Isolates that grew on selective media were then streaked onto brain-heart infusion (BHI) agar amended with the selective antibiotic for each organism to isolate colonies for species (*E. coli*) or genus (*Enterococcus*) confirmation. Each isolate was confirmed using qPCR, *E. coli* by a *uid*A species-specific assay [65] and *Enterococcus* spp. by a genus-specific *Enterococcus* assay (Entero1a) [66]. If the isolated colonies were confirmed to be the appropriate genus or species, they were then placed into a BHI broth

amended with the selective antibiotic, ampicillin (16 µg/mL) for *E. coli* and erythromycin (4 µg/mL) for *Enterococcus* spp., overnight. To ensure adequate long-term storage, 900 µL of the overnight culture was combined with 900 µL of a 60% glycerol stock into a sterile cryovial amended with the selected antibiotic and stored in a -80°C freezer for future multidrug resistance testing.

Multidrug Resistance Testing

Colonies whose species (*E. coli*) or genus (*Enterococcus*) were confirmed through qPCR were also confirmed for antibiotic resistance to the selection antibiotic, and for resistance to other antibiotics using the Kirby-Bauer disc diffusion assay [67]. Archived isolates were resuscitated on BHI agar amended with the selection antibiotic and allowed to incubate overnight at 35°C for *E. coli* and *Enterococcus* spp.. These colonies were then inoculated into a 2 mL microcentrifuge tube containing 1 mL of sterile saline creating a suspension equivalent to the turbidity of a 0.5 McFarland standard, which was verified by Nanodrop at wavelength of 625 nanometers [67]. A sterile swab was then be dipped into the microcentrifuge tube and used to inoculate 150 mL Mueller-Hinton agar plates by streaking the swab over the entire surface, ensuring even distribution of inoculum. Using flame sterilized forceps, the selected antibiotic disks for *E. coli* and *Enterococcus* spp. (Table 1) were applied to the inoculated media [11,67]. Plates were then incubated for 24 hours at 35°C for both organisms [67] and the diameter of the zone of inhibition (the area of clearing in the bacterial lawn around each disc) was recorded to the nearest millimeter. The zone of inhibition diameter was compared to the Clinical and Laboratory Standards Institute (CLSI)[11] document to gauge susceptibility to each antibiotic.

Statistical Analysis

E. coli and enterococci concentrations were $\log_{10}$ transformed for statistical analysis. Two-way ANOVA followed by Tukey's multiple comparison test for both *E. coli* and enterococci were performed on individually to determine if there were significant differences in bacterial concentrations among beaches or among subsites. Analyses were performed using GraphPad Prism version 9.3.1 for (GraphPad Software, San Diego, California USA, www.graphpad.com). Chi squared tests were performed to determine differences in frequency of antibiotic resistant (1) *E. coli* and (2) enterococci, and the frequency of multidrug resistance of (1) *E. coli* and (2) *Enterococcus* spp. among the 3 different beaches and subsites.

Table 1. Antibiotics chosen for *E. coli* and *Enterococcus* spp. to be used in Kirby-Bauer disc diffusion assay and their clinical relevance.

NAME (ABBREVIATION)	CLASS	CLINICAL RELEVANCE	SPECIES
AMOXICILIN CLAVULANATE (AMC)	β -Lactam/β-Lactamase Inhibitor	Highly prescribed in the US [68]	EC
CEFOTAXIME (CTX)	Cephems	Prescribed widely	EC
FOSFOMYCIN (FOS)	Fosfomycin's	Used as a primary treatment for *E. coli* infections that express multidrug resistance [71]	EC
GENTAMICIN (CN)	Aminoglycosides	Widely used against Gram-negative bacteria [72]	EC
IMIPENEM (IMP)	Carbapenems	Considered reliable for treating infections [73] although resistance has been expressed [70]	EC
AMPICILLIN (AMP)	Penicillin's	Used clinically as a primary drug [74]. *E. coli* Isolates were initially selected for intermediate resistance and now with Kirby Bauer were checked for full resistance	EC, ENT
CIPROFLOXACIN (CIP)	Fluoroquinolones	Broad spectrum antibiotic used for both gram-positive and gram-negative infections[70,75]	EC, ENT
ERYTHROMYCIN (E)	Macrolides	The most commonly prescribed antibiotics worldwide [33,34]. *Enterococcus* spp. Isolates were initially selected for intermediate resistance and now with Kirby Bauer were checked for full resistance	ENT
LINEZOLID (LZD)	Oxazolidinones	Used in multidrug resistant infections such as bacteremia associated with vancomycin-resistant *Enterococcus faecium* [76]	ENT
TETRACYCLINE (TE)	Tetracyclines	Used as a broad-spectrum treatment, for Gram-positive and negative bacteria [77]	ENT
VANCOMYCIN (VA)	Glycopeptides	Used for expressed β -lactams resistance [78]	ENT

*EC: included in E. coli panel; ENT: included in Enterococcus spp. panel

RESULTS

Primary Isolation of Antibiotic-Resistant Fecal Indicator Bacteria

E. coli

Beaches. Environmental conditions among the three beaches did not vary significantly (Table A1). Among the three beaches there was no significant difference in total *E. coli* concentrations ($P = 0.14$) (Table 2). Mean $\log_{10}$ *E. coli* concentrations (CFU/100 g or 100 ml) were 1.82 at Ben T. Davis, 2.61 at Courtney Campbell, and 1.78 at Cypress Point.

Thirty percent of *E. coli* expressed intermediate phenotypic ampicillin resistance, and concentrations were significantly different among beaches *(*$P = 0.02$). Courtney Campbell Beach (mean $\log_{10}$ 0.96 CFU/100g or 100 mL) had a significantly higher concentration of ampicillin-resistant *E. coli* compared to Cypress Point Park ($P = 0.02$) while concentrations at Ben T. Davis did not differ significantly from either beach. Proportions of ampicillin-resistant *E. coli* were significantly higher at Ben T. Davis ($P = 0.0001$) and Courtney Campbell beach ($P = 0.0001$) compared to Cypress Point Park.

Subsites. Total *E. coli* concentrations among subsites (water, swash zone, 2.5 m away from the swash zone) were also not significantly different ($P = 0.44$) ranging from mean $\log_{10}$ concentrations (CFU/100 g or 100 ml) of 1.87 in the water, 1.94 in the swash zone sand, and 2.41 in the sand 2.5 m away from the swash zone (Figure 2). Of the ampicillin-resistant *E. coli* there was a significant difference in concentration among the three subsites ($P = 0.03$). The highest concentration of ampicillin-resistant *E. coli* among subsites were observed 2.5 m away from the swash zone (mean $\log_{10}$ 1.0

CFU/100 g), which was significantly higher than the water (mean $\log_{10}$ 0.2 CFU/100 mL) (P = 0.02) while the measurements in the swash zone did not differ significantly from either subsite (Figure 2). The overall proportion of ampicillin-resistant *E. coli* were significantly higher in the swash zone (P = 0.0001) and 2.5 meters away from the swash zone (P = 0.01) compared to the water (Figure 3).

Enterococci

Beaches. Enterococci concentrations were significantly different among beaches (P = 0.02) (Table 2). Mean $\log_{10}$ enterococci concentrations (CFU/100 g or 100 ml) were 3.08 at Ben T. Davis, 3.09 at Courtney Campbell, and 2.29 at Cypress Point. Courtney Campbell Beach and Ben T. Davis Beach both had significantly higher concentration of enterococci (P = 0.04) than Cypress Point Park. Enterococci concentrations in water were compared to marine beach action values (BAV) which are determined by USEPA. Among the three beaches Ben T. Davis, Courtney Campbell, and Cypress Point all exceeded the BAV at 66%, 100% and 33% of the sampling events respectively (Figure 4).

Forty-one percent of enterococci expressed intermediate erythromycin-resistance with the highest concentrations observed at Courtney Campbell beach (mean $\log_{10}$ 1.42 CFU/100g or 100 mL) with no significant difference (P = 0.35) among beaches observed. Proportions of erythromycin-resistant enterococci were significantly higher at Cypress Point Park compared to Ben T. Davis (Figure 5).

Subsites. Total enterococci concentrations were significantly different among subsites (P = 0.006) ranging from Mean $\log_{10}$ concentrations (CFU/100 g or 100 ml) of 2.17 in the water, 3.22 n the swash zone sand, and 3.06 in the sand 2.5 m away from the swash zone. Total enterococci in swash zone sand (P = 0.007) and 2.5 m away from the

swash zone (P = 0.02) were greater than those in water, with the swash zone having the highest levels overall (mean $\log_{10}$ 3.22 CFU/100g) (Figure 4). Differences in erythromycin-resistant enterococci among subsites was not significant (P = 0.06), with the highest levels being 2.5 m away from the swash zone (mean $\log_{10}$ 1.49 CFU/100g) (Figure 4). Proportions of erythromycin-resistant enterococci were significantly higher 2.5 meters away from the swash zone compared to water (P = 0.04) (Figure 5).

Phylogenetic Confirmation of Antibiotic-Resistant *E. coli* and *Enterococcus* spp.

Up to 15 resistant isolates per sub-site (5 per sampling event) were picked for multiple-drug resistance testing and were confirmed to species in the case of *E. coli* or genus in the case of *Enterococcus* spp. (Table 3). Seventy-three *E. coli* isolates out of 75 (97%) were confirmed to species and 93 out of 112 enterococci (83%) were confirmed *Enterococcus* spp.. All confirmed isolates were subsequently tested for multidrug resistance.

Multidrug-Resistance Testing of Ampicillin-Resistant *E. Coli* and Erythromycin-Resistant *Enterococcus* spp.

E. coli

Seventy-three ampicillin-resistant *E. coli* isolates were tested for multi-drug resistance. Of these isolates, 100% expressed full resistance to ampicillin and were less frequently resistant to ciprofloxacin (34.3%), gentamicin (26.0%), cefotaxime (17.8%), fosfomycin (2.7%), amoxicillin/clavulanate (1.4%) and imipenem (1.4%) (Figure 6). Fifty-five percent (n = 40) of all ampicillin-resistant *E. coli* isolates were resistant to at least one other antibiotic and 18.2% (n = 15) expressed multidrug resistance patterns.

Table 2. Two-way ANOVA percent variability and *P*-values of total and ampicillin-resistant *E. coli* and of total and erythromycin-resistant enterococci. Significant values are bolded.

Organism	**Location**					
	Beach		Subsite		Interaction	
	% Variability	*P*-value	% Variability	*P*-value	% Variability	*P*-value
E. coli	17.31	.14	6.79	.44	4.22	.90
Ampicillin-Resistant *E. coli*	24.11	**.02**	22.00	**.03**	10.10	.42
Enterococci	21.83	**.02**	33.12	**.006**	2.31	.91
Erythromycin-Resistant Enterococci	6.66	.35	20.22	.057	19.20	.21

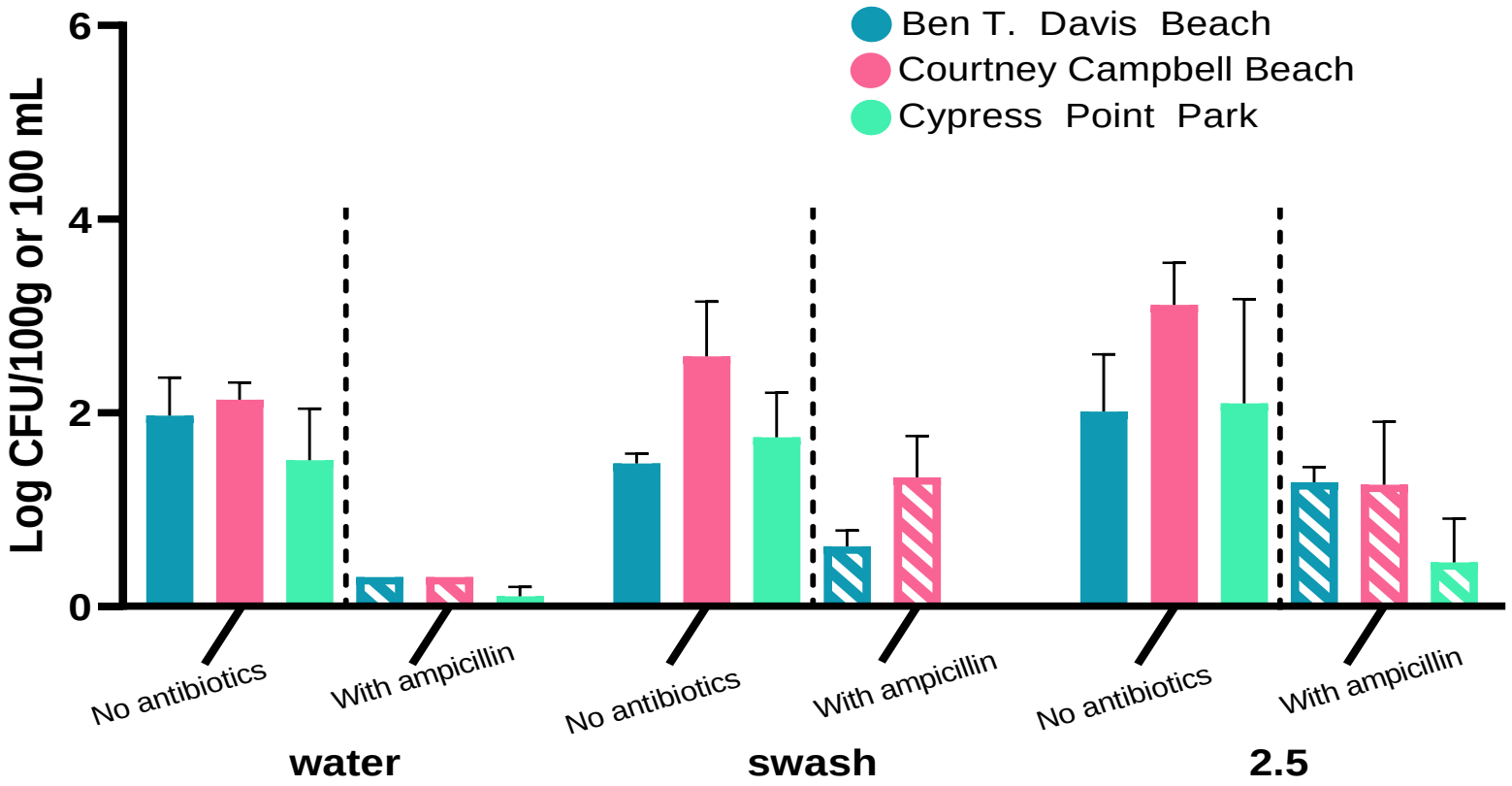

Figure 2. The mean $\log_{10}$ transformed CFU/100 g (sand) or 100 mL (water) of total and ampicillin-resistant *E. coli* at each beach. Error bars represent standard error of the mean.

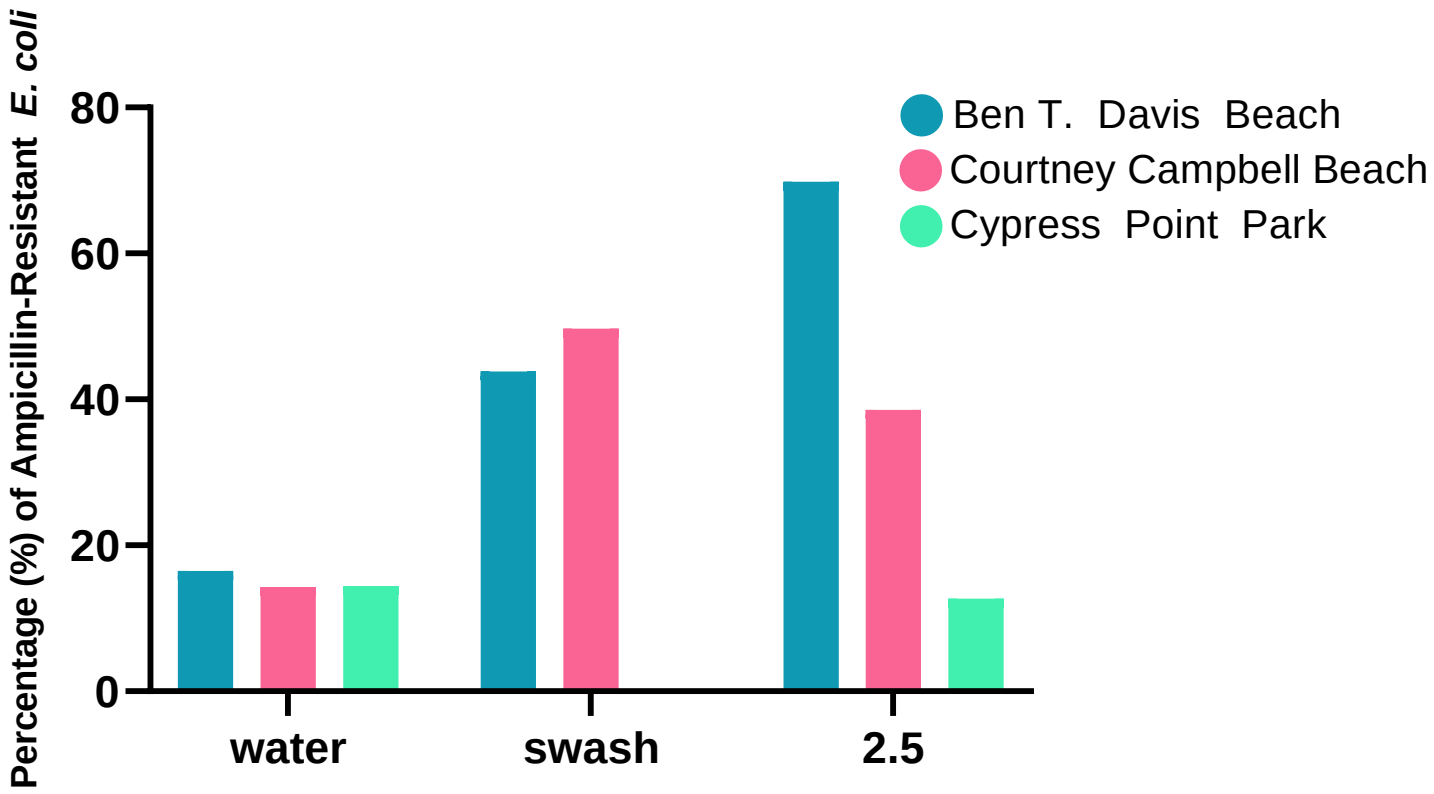

Figure 3. The proportion (%) of ampicillin-resistant E. coli among beaches and subsites.

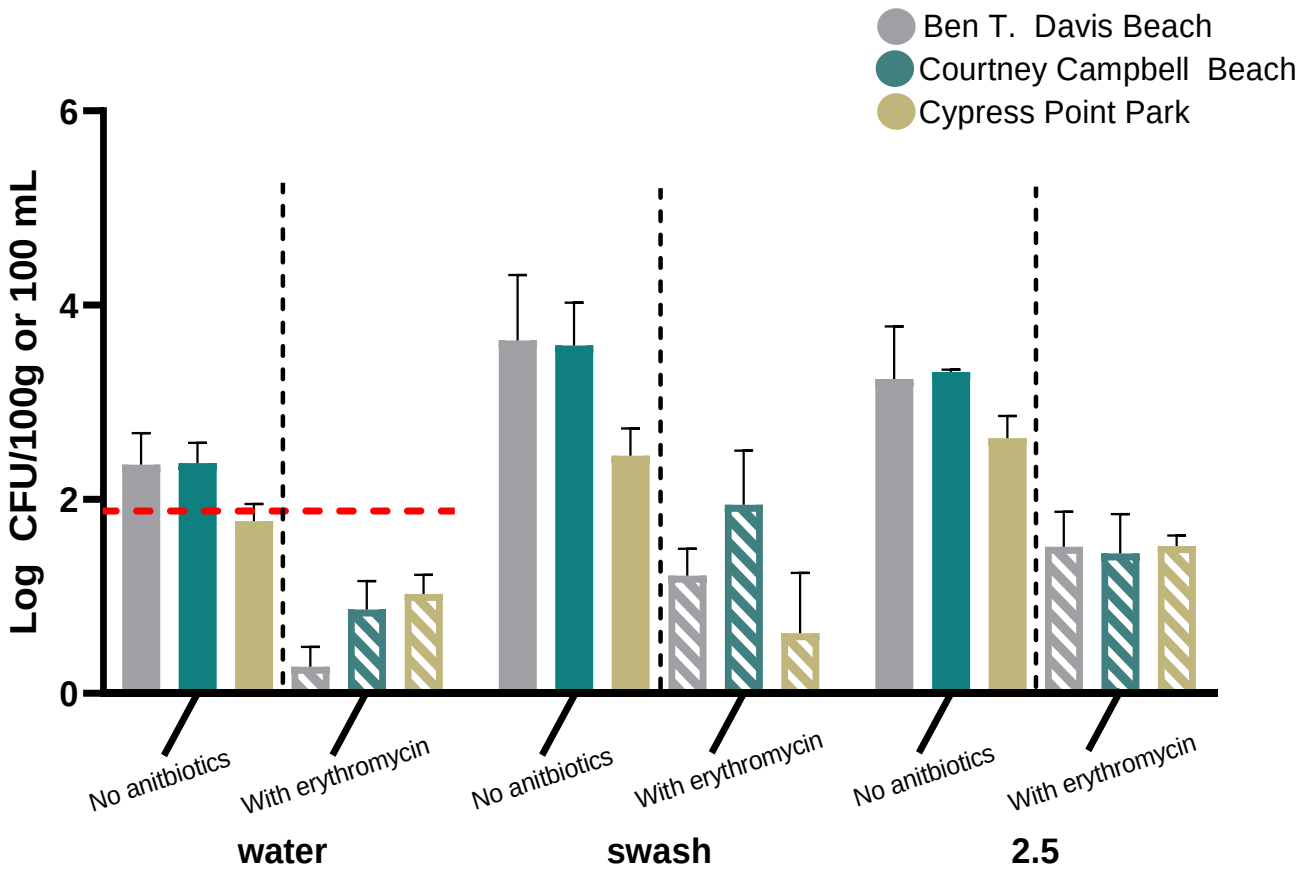

Figure 4. The mean $\log_{10}$ transformed CFU/100 g (sand) or /100 mL (water) of total and erythromycin-resistant enterococci at each beach. Red dashed line indicates the US EPA beach action value and error bars represent standard error of the mean.

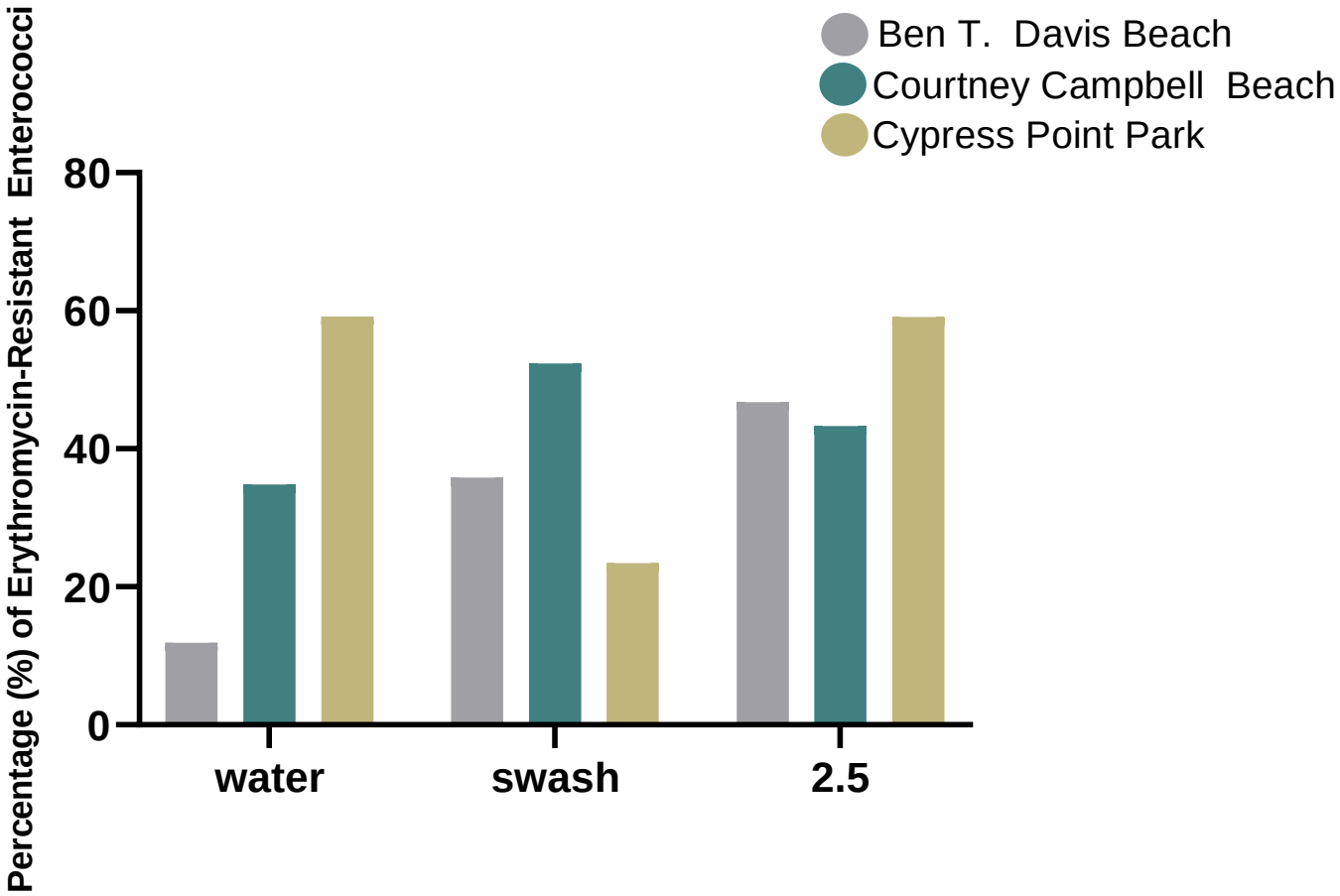

Figure 5. The proportion (%) of erythromycin-resistant enterococci among beaches and subsites.

Table 3. Number and percentage of antibiotic-resistant *E. coli* and *Enterococcus* spp. isolates confirmed from primary isolation and further tested for multi-drug resistance.

Site	Sub-Site	Ampicillin-Resistant Isolates Confirmed as *E. coli*	Erythromycin-Resistant Isolates Confirmed as *Enterococcus* spp.
Ben T Davis	**Water**	13 (100%)	14 (93%)
	Swash	7 (88%)	10 (91%)
	2.5 m	12 (100%)	10 (77%)
Courtney Campbell	**Water**	15 (100%)	14 (93%)
	Swash	7 (100%)	11 (100%)
	2.5 m	9 (90%)	8 (100%)
Cypress Point Park	**Water**	5 (100%)	11 (73%)
	Swash	0	7 (78%)
	2.5 m	5 (100%)	8 (53%)
Total		73 (97%)	93 (83%)

*No ampicillin-resistant E. coli colonies were observed at Cypress Point Park in the swash zone.

Of the MDR isolates, all expressed full resistance to the selective antibiotic, ampicillin, and ciprofloxacin and were less frequently resistant to cefotaxime (73.3%), gentamicin (53.3%), amoxicillin/clavulanate (6.7%), imipenem (6.7%) and fosfomycin (0%) (Table 4). The most frequent MDR pattern observed (46.7% of ampicillin-resistant isolates) was to ampicillin, ciprofloxacin, and cefotaxime, followed by a pattern expressed by 26.7% of ampicillin-resistant isolates: ampicillin, ciprofloxacin, and gentamicin.

Among beaches, the proportion of MDR *E. coli* were significantly different (P = 0.0271). The difference in frequency of MDR *E. coli* was significantly lower at Courtney Campbell Beach compared to Ben T. Davis (P = 0.0433) and Cypress Point Park (P = 0.0235) (Figure 7a). Among subsites the frequency of MDR *E. coli* was significantly higher 2.5 m away from the swash zone compared to the swash zone (P = 0.0158) (Figure 7b) and there was no significant difference among water compared to either subsite.

Enterococcus spp.

Ninety-three erythromycin-resistant *Enterococcus* spp. isolates were tested for multi-drug resistance. Of these isolates, 95% showed full resistance to erythromycin. Other resistance phenotypes were observed in the following order: tetracycline (58.1%), ciprofloxacin (37.6%), ampicillin (36.6%), linezolid (31.2%), and vancomycin (11.8%) (Figure 8). Eighty-three percent (n = 77) of all isolates showed resistance to at least one other antibiotic and 53.4% (n = 50) expressed multidrug resistance patterns. Of the MDR isolates, all expressed full resistance to the selective antibiotic, erythromycin and were less frequently resistant to tetracycline (88.0%) followed by ciprofloxacin (64.0%), linezolid (54.0%), ampicillin (44.0%), and vancomycin (20.0%). The most frequent MDR patterns observed were among erythromycin and tetracycline combined with either ampicillin (16.0%), ciprofloxacin (14.0%) or linezolid (14.0%%) (Table 5). All MDR isolates that expressed vancomycin resistance were also resistant to tetracycline and 70% expressed resistance to at least 4 antibiotics.

No significant differences in concentration of MDR *Enterococcus* spp. were observed among beaches (P = 0.7537). Frequencies of MDR *Enterococcus* spp. ranged

from 50.0%-58.5% with the highest observed frequency at Ben T. Davis (Figure 9a). There was also no significant difference in concentration of MDR isolates among subsites (P = 0.8788). Frequencies of MDR *Enterococcus* spp. ranged from 51.3%-57.7%with 2.5 meters away from the swash zone having the highest overall frequency (Figure 9b).

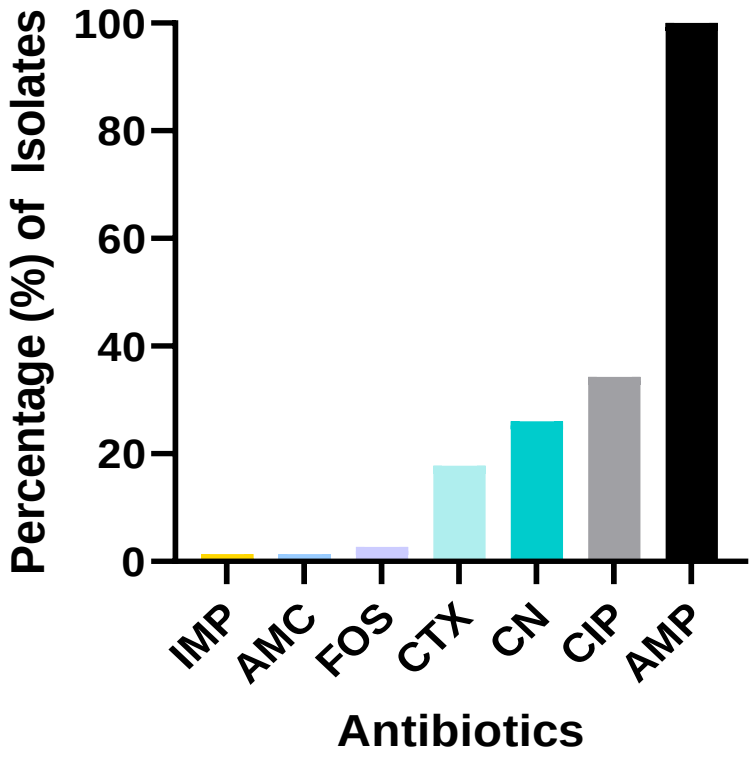

Figure 6. Frequency of full resistance to seven classes of antibiotics among ampicillin-resistant *E. coli*(n=73), including ampicillin, the selective antibiotic, among bacteria isolated from sand and water. Ampicillin – AMP, ciprofloxacin – CIP, gentamicin – CN, cefotaxime – CTX, fosfomycin -FOS, amoxicillin/clavulanate-AMC, imipenem – IMP.

Table 4. Multi-drug resistance profiles of *E. coli* isolates (n = 15). Antibiotics arranged by percent resistance. White boxes indicate susceptibility, yellow boxes indicate intermediate resistance, and red boxes indicate full resistance.

Site	Subsite	AMP	CIP	CTX	CN	AMC	IMP	FOS	% of Isolates
CCB	W	R	R	R	R	R	R	S	6.7%
BTD	W	R	R	R	R	S	S	S	20.0%
CPP	2.5	R	R	R	R	S	S	S	
CPP	2.5	R	R	R	R	S	S	S	
BTD	W	R	R	S	R	S	S	S	26.7%
BTD	W	R	R	S	R	S	S	S	
BTD	2.5	R	R	S	R	S	S	S	
CPP	2.5	R	R	S	R	S	S	S	
BTD	W	R	R	R	S	S	S	S	46.7%
BTD	2.5	R	R	R	S	S	S	S	
BTD	2.5	R	R	R	S	S	S	S	
BTD	2.5	R	R	R	S	S	S	S	
BTD	2.5	R	R	R	S	S	S	S	
CCB	W	R	R	R	I	S	S	S	
CPP	2.5	R	R	R	S	S	S	S	

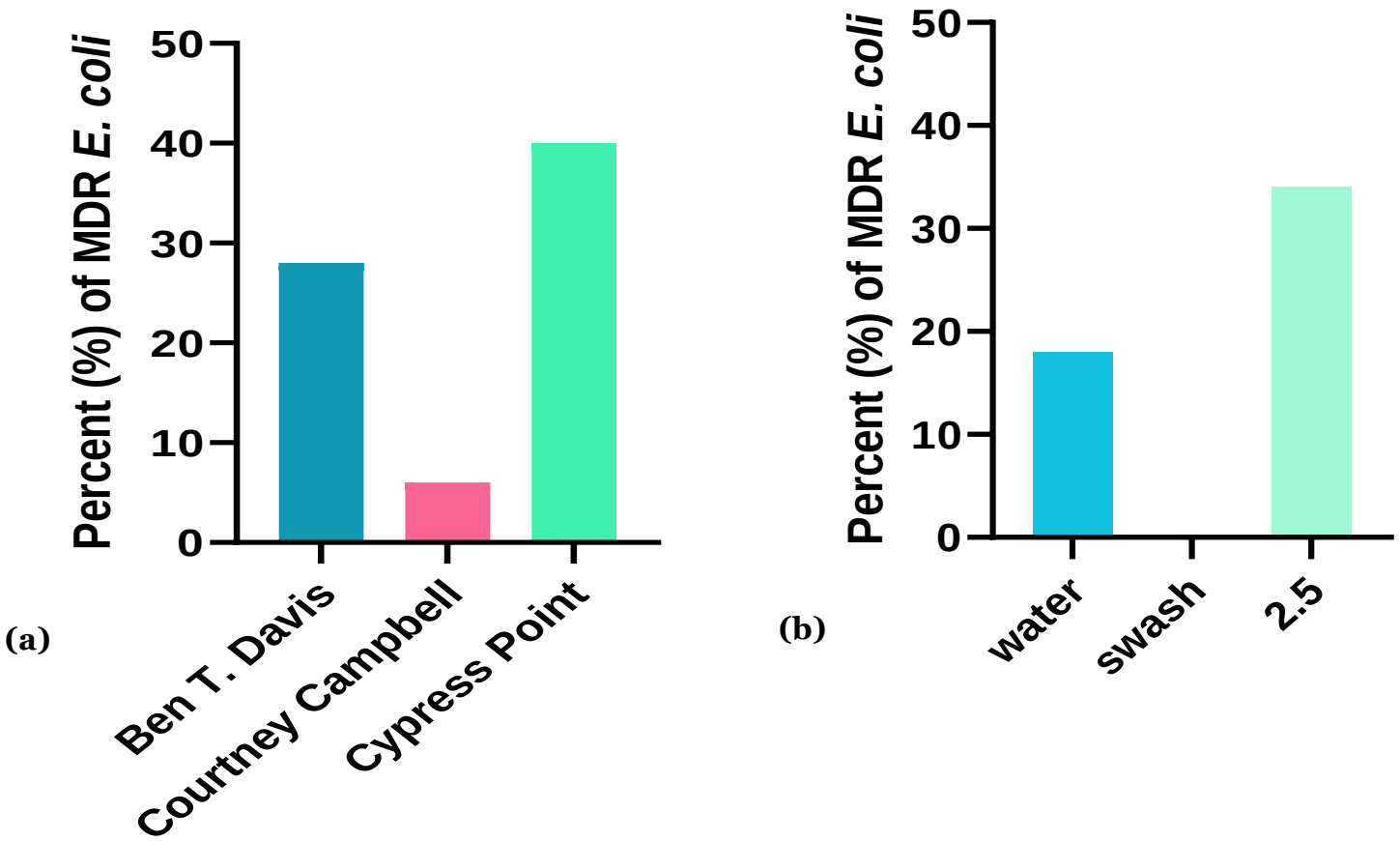

Figure 7. Frequency of multi-drug resistant *E. coli* isolates in the ampicillin-resistant population. Among **(a)** beaches (n=9, 2, & 4 respectively), and **(b)** subsites (n=6, 0, & 9 respectively).

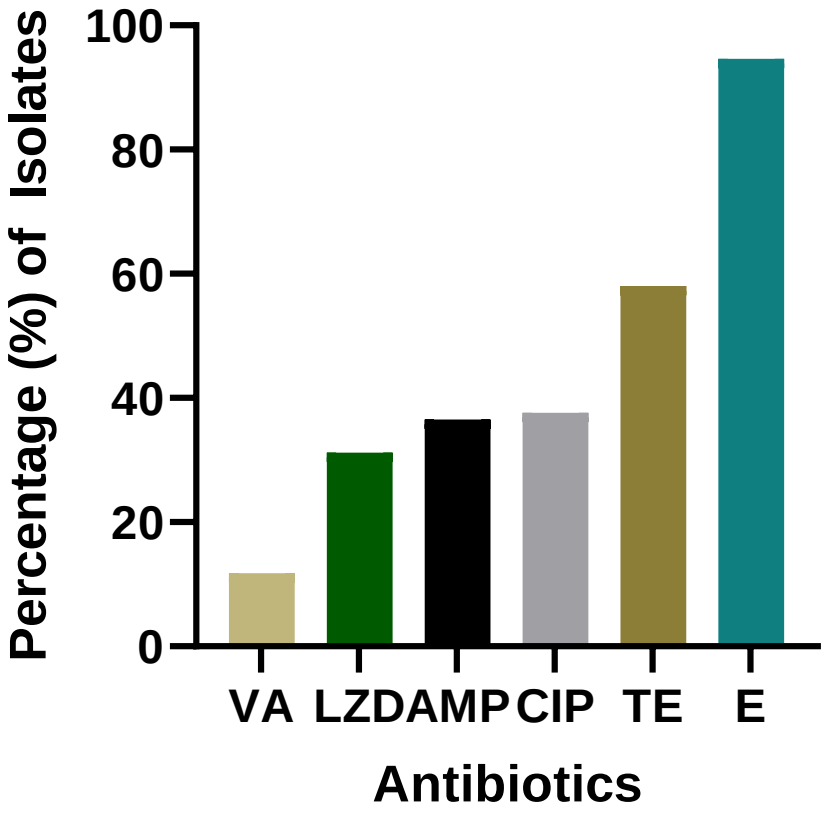

Figure 8. Frequency of full resistance to six classes of antibiotics among erythromycin-resistant *Enterococcus* spp. (n=93), including erythromycin, the selective antibiotic, among bacteria isolated from sand and water. Erythromycin – E, tetracycline – TE, ciprofloxacin – CIP, ampicillin – AMP, linezolid – LZD, vancomycin – VA.

Table 5. Multi-drug resistance profiles of erythromycin-resistant *Enterococcus* spp. isolates (n=50). Antibiotics arranged by percent resistance. White boxes indicate susceptibility, yellow boxes indicate intermediate resistance, and red boxes indicate full resistance.

Site	Subsite	E	TE	CIP	LZD	AMP	VA	% of Isolates
CPP	SWSH	R	R	I	R	R	I	2.0%
BTD	W	R	R	R	S	R	R	2.0%
CCB	SWSH	R	R	R	R	R	R	2.0%
BTD	W	R	S	R	S	R	S	4.0%
CCB	W	R	S	R	S	R	I	
BTD	2.5	R	I	R	R	S	I	4.0%
CCB	SWSH	R	S	R	R	S	S	
CCB	W	R	I	R	R	R	I	4.0%
CPP	SWSH	R	S	R	R	R	I	
CCB	2.5	R	R	R	S	R	S	4.0%
CCB	2.5	R	R	R	S	R	S	
BTD	2.5	R	R	S	R	R	R	4.0%
CCB	SWSH	R	R	S	R	R	R	
BTD	SWSH	R	R	R	I	S	R	6.0%
CCB	2.5	R	R	R	S	S	R	
CPP	W	R	R	R	I	S	R	
BTD	2.5	R	R	R	R	R	S	6.0%
CPP	W	R	R	R	R	R	S	
CPP	2.5	R	R	R	R	R	I	
BTD	W	R	R	R	R	S	R	6.0%
BTD	W	R	R	R	R	S	R	
BTD	SWSH	R	R	R	R	S	R	

Site	Subsite	E	TE	CIP	LZD	AMP	VA	% of Isolates
BTD	SWSH	R	R	R	R	S	I	12.0%
BTD	2.5	R	R	R	R	S	I	
BTD	2.5	R	R	R	R	S	I	
CPP	W	R	R	R	R	S	I	
CPP	W	R	R	R	R	S	I	
CPP	2.5	R	R	R	R	S	I	
BTD	W	R	R	R	S	S	I	14.0%
BTD	W	R	R	R	S	S	I	
BTD	SWSH	R	R	R	S	S	I	
BTD	SWSH	R	R	R	S	S	I	
CCB	2.5	R	R	R	S	S	I	
CPP	W	R	R	R	I	S	I	
CPP	2.5	R	R	R	S	S	S	
BTD	W	R	R	I	R	S	I	14.0%
BTD	2.5	R	R	S	R	S	I	
CCB	W	R	R	S	R	S	I	
CCB	W	R	R	I	R	S	I	
CCB	SWSH	R	R	I	R	S	I	
CCB	SWSH	R	R	I	R	S	I	
CPP	W	R	R	S	R	S	I	
BTD	W	R	R	I	I	R	I	16.0%
BTD	SWSH	R	R	I	I	R	I	
CCB	W	R	R	S	I	R	I	
CCB	SWSH	R	R	I	I	R	I	
CCB	SWSH	R	R	I	I	R	I	
CCB	2.5	R	R	I	S	R	I	
CPP	W	R	R	I	I	R	I	
CPP	2.5	R	R	I	I	R	S	

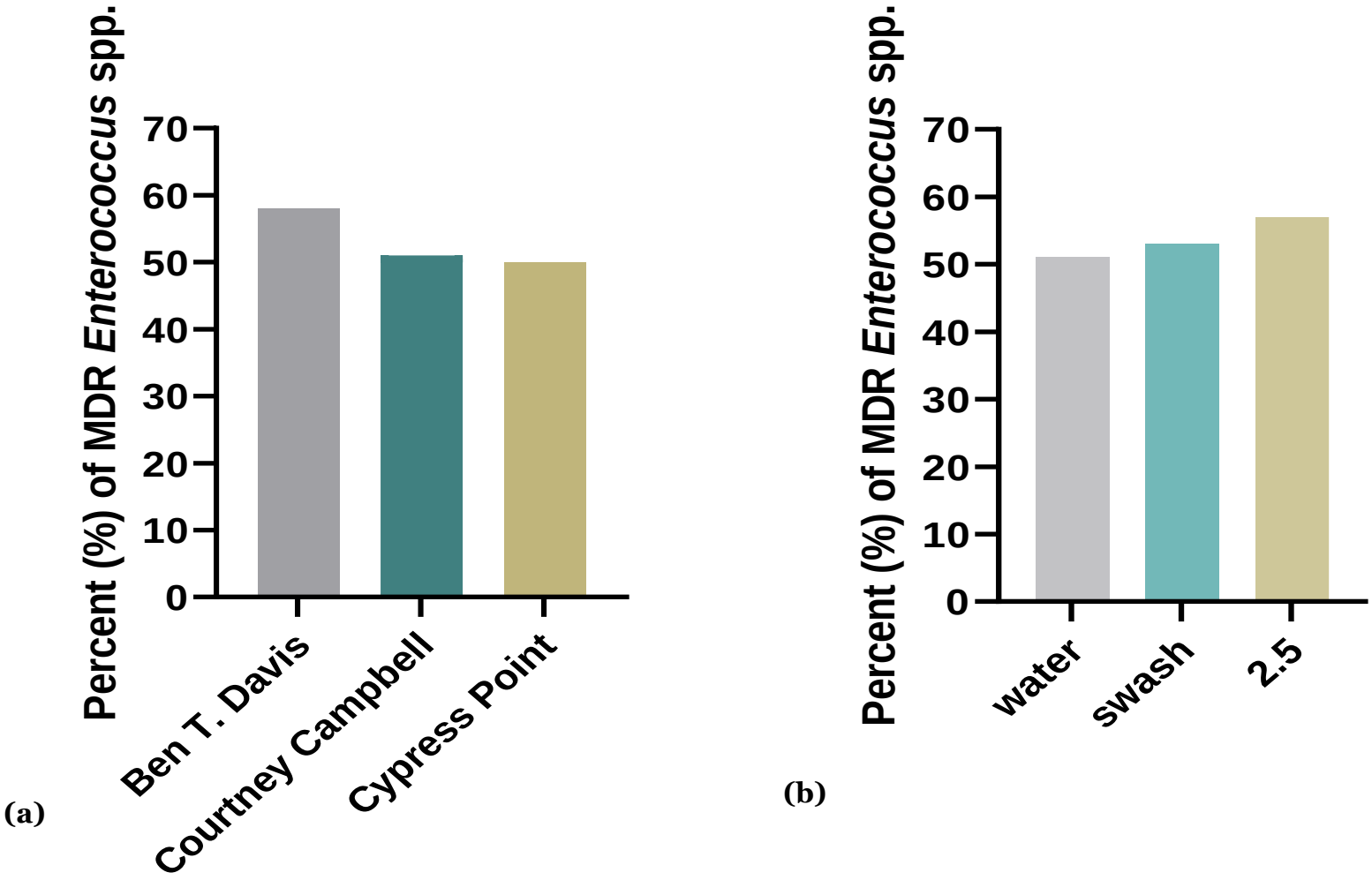

Figure 9. Frequency of multi-drug resistance among erythromycin-resistant *Enterococcus* spp. Isolates. Among **(a)** beaches (n=20, 17, & 13 respectively), and **(b)** subsites (n= 20, 15 & 15 respectively).

DISCUSSION

The role of sand as a reservoir for antibiotic-resistant FIB remains unclear. Gaining a better understanding of the distribution of antibiotic-resistant FIB in sand is necessary to understand the public health implications of exposure to beach sand. This study was intended to compare the levels of single and multidrug-resistant *E. coli* and *Enterococcus* spp. between three beach areas where beachgoers could be exposed. Unlike most other studies of antibiotic-resistant FIB at beaches, this study compared bacterial levels at three distinct areas of potential human exposure at the beach: water, swash zone sand, and foreshore sand 2.5 m from the swash zone.

Contribution of Beach Area to FIB Levels

Beach location. Geographic location of the beaches contributed to significant differences in overall concentration of *E. coli* and enterococci and proportion of resistance to the selective antibiotic. The significantly higher concentrations and proportions of ampicillin-resistant *E. coli* observed at Courtney Campbell Beach compared Cypress Point Park coincide with significantly higher concentrations of total enterococci at Courtney Campbell beach compared to Cypress Point Park. Dogs are permitted at Courtney Campbell Beach, but not at the other two beaches, which could be a factor in elevated FIB levels. The impact of dog feces at beaches has been documented in California studies, which found that pet waste can be a significant source of fecal

contamination [79,80] .Another contributing factor could be the lack of buffer zone between the sand and the highway.

Trends in proportion of multidrug-resistant *E. coli* and enterococci did not follow the same trends as their single resistant varieties. Significantly higher proportions of ampicillin-resistant *E. coli* were observed in Courtney Campbell Beach compared to Cypress Point Park, however there were significantly higher proportions of multidrug-resistant *E. coli* at Cypress Point Park compared to Courtney Campbell Beach. The Florida Healthy Beaches program did not report any FIB exceedances at Cypress Point Park during these sampling events. No obvious impacts on the beach were noted that would have caused the observed elevated levels of antibiotic-resistant bacteria.

Subsite location. In many cases subsites contributed to differences in total and antibiotic-resistant *E. coli* and enterococci levels, most notably between 2.5 m away from the swash zone and the water. Ampicillin-resistant *E. coli* concentrations were significantly higher 2.5 meters away from the swash zone compared to water at all three beaches. Similar patterns were observed between total enterococci and erythromycin-resistant enterococci concentrations (at $\alpha = 0.1$), which were significantly higher 2.5 meters away from the swash zone compared to in the water. Frequencies of both ampicillin-resistant *E. coli* and erythromycin-resistant enterococci increased in proportion moving from the water towards 2.5 meters away from the swash zone.

Trends in proportion of multidrug-resistant *E. coli* and *Enterococcus* spp. among subsites followed trends observed for their single resistant varieties. Frequencies of MDR *E. coli* and MDR *Enterococcus* spp. exhibited differences among the subsites; the highest frequency was 2.5 meters away from the swash zone. This trend is consistent

with results from the single drug resistant varieties of *E. coli* and *Enterococcus* spp. This observation suggests that exposure to foreshore sand 2.5 m from the swash zone could increase the likelihood that beach recreators in the sandy areas of the beach would encounter MDR pathogens.

Patterns of Resistance

Multidrug resistance was prevalent in *E. coli* and *Enterococcus* spp. that were isolated from both sand and water. All MDR *E. coli* isolates expressed resistance to both ampicillin and ciprofloxacin, which was also seen in study of a freshwater lake, where ampicillin resistance was observed in 12.9% of the population compared to 30% in this study [14]. The most common pattern of resistance among the MDR *E. coli* isolates was to ampicillin, ciprofloxacin, and cefotaxime, and of these 36.4% were resistant to at least one other antibiotic including imipenem. Imipenem is a type of carbapenem, a drug of last resort and the occurrence of these resistance genes has been observed in the environment by Ahmed et al. in storm water runoff within the Tampa Bay area [82]. Ampicillin and ciprofloxacin are commonly prescribed broad-spectrum antibiotics used to treat infections; therefore, one could anticipate a high frequency of resistance to these antibiotics if the FIB originate from sewage [70,75].

In this study, multidrug-resistant *Enterococcus* spp. were more frequently observed than MDR *E. coli*. All of the MDR *Enterococcus* spp. were resistant to erythromycin and 88% showed resistance to tetracycline. Resistance to erythromycin, tetracycline, and ampicillin was the most frequently observed pattern and occurred at all three beaches in sand and water. Vancomycin resistance was also observed in 20% of

the MDR isolates, and all of these isolates also expressed resistance to tetracycline and erythromycin. The greatest proportion of vancomycin resistance occurred at Ben T. Davis Beach, with 60% of all vancomycin-resistant isolates observed here.

Reports of vancomycin-resistant *Enterococcus* spp. in the environment are still relatively uncommon. A small number of studies have reported intermediate and fully vancomycin-resistant *Enterococcus* spp. in freshwater beaches including the sand matrix [20,83]. The concern of FIB expressing resistance to antibiotics, especially broad-spectrum antibiotics, is that they will pass on these resistance genes to other susceptible or single resistant bacteria in sand and also for their potential to infect beachgoers [14].

During this study, recreational estuarine beaches, including sand, harbor both single and multidrug-resistant *E. coli* and *Enterococcus* spp. Antibiotic-resistant bacteria were also more concentrated in sand than in water, an observation that has been corroborated by other research [53,84,85]. The increased frequency of *E. coli* and *Enterococcus* spp. in the sand may be explained due to more favorable conditions in the sand environment including availability of nutrients, and protection from UV radiation and predation [50,86,87].

The spatial variability and proximity to anthropogenic factors of the three beaches may also have played a role in the concentration of single and multidrug-resistant *E. coli* and *Enterococcus* spp. Beaches with little to no buffer zones from highways and urbanization, like Courtney Campbell Beach and Ben T. Davis beach, both exceeded beach action values of enterococci more frequently and both had significantly greater concentrations of ampicillin-resistant *E. coli* and erythromycin-resistant enterococci compared to Cypress Point Park. Similar results were observed in other

studies showing that areas with high anthropogenic pollution are correlated with an increased presence of antibiotic resistance [88,89]. However, Cypress Point Park, which is the least impacted beach in this study judging from surrounding infrastructure and impervious surface, had a significantly higher proportion of MDR *E. coli* compared to Courtney Campbell Beach and a significantly higher proportion of erythromycin-resistant enterococci compared to Ben T. Davis Beach. Further research would be necessary to provide an explanation for this observation, but it could be due to undiscovered sources of sewage.

One limitation of this study was the small sample size. More sampling events could have increased the significance of values that were marginally non-significant, specifically the distribution of erythromycin-resistant enterococci among subsites. Future studies should include microbial source tracking to determine the origin of FIB expressing antibiotic resistance to assess the overall risk of contact with human-related pathogens.

Having a better understanding of sand as a reservoir for antibiotic-resistant FIB is crucial as the implications of increased antibiotic resistance could have the potential to lead to more antibiotic-resistant pathogens. These antibiotic-resistant pathogens could cause serious health problems in beach goers as well as lead to the increased spread of antibiotic-resistant genes into other environments beyond the beach.

REFERENCES

1. *Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report 2021.*; 2021.
2. US Department of Health and Human Services, CDC. Antibiotic Resistance Threats in the United States. *Centers for Disease Control and Prevention*. Published online 2019:1-113.
3. Sarmah AK, Meyer MT, Boxall ABA. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere*. 2006;65(5):725-759. doi:10.1016/j.chemosphere.2006.03.026
4. Solo-Gabriele HM, Harwood VJ, Kay D, et al. Beach sand and the potential for infectious disease transmission: Observations and recommendations. *Journal of the Marine Biological Association of the United Kingdom*. 2016;96(1):101-120. doi:10.1017/S0025315415000843
5. Cantón R, Morosini MI. Emergence and spread of antibiotic resistance following exposure to antibiotics. *FEMS Microbiology Reviews*. 2011;35(5):977-991. doi:10.1111/j.1574-6976.2011.00295.x
6. Brunton LA, Desbois AP, Garza M, et al. Identifying hotspots for antibiotic resistance emergence and selection, and elucidating pathways to human exposure: Application of a systems-thinking approach to aquaculture systems. *Science of the Total Environment*. 2019;687:1344-1356. doi:10.1016/j.scitotenv.2019.06.134
7. Kennedy K, Collignon P. Colonisation with *Escherichia coli* resistant to "critically important" antibiotics: A high risk for international travellers. *European Journal of Clinical Microbiology and Infectious Diseases*. 2010;29(12):1501-1506. doi:10.1007/s10096-010-1031-y
8. Leonard AFC, Singer A, Ukoumunne OC, Gaze WH, Garside R. Is it safe to go back into the water? A systematic review and meta-analysis of the risk of acquiring infections from recreational exposure to seawater. *International Journal of Epidemiology*. 2018;47(2):572-586. doi:10.1093/IJE/DYX281
9. Jiang L, Hu X, Yin D, Zhang H, Yu Z. Occurrence, distribution and seasonal variation of antibiotics in the Huangpu River, Shanghai, China. *Chemosphere*. 2011;82(6):822-828. doi:10.1016/j.chemosphere.2010.11.028
10. Andersson DI, Nicoloff H, Hjort K. Mechanisms and clinical relevance of bacterial heteroresistance. *Nature Reviews Microbiology*. 2019;17(8):479-496. doi:10.1038/s41579-019-0218-1
11. CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 26th ed. CLSI supplement M100S. Wayne, PA: Clinical and Laboratory Standards Institute; 2016.

12. World Health Organization. *Global Antimicrobial Resistance Surveillance System (GLASS) Report*.; 2018.
13. Sahloff EG, Martin SJ. *Resistance in the ICU*.; 2011.
14. Alm EW, Zimbler D, Callahan E, Plomaritis E. Patterns and persistence of antibiotic resistance in faecal indicator bacteria from freshwater recreational beaches. *Journal of Applied Microbiology*. 2014;117(1):273-285. doi:10.1111/jam.12512
15. Blaak H, De Kruijf P, Hamidjaja RA, Van Hoek AHAM, De Roda Husman AM, Schets FM. Prevalence and characteristics of ESBL-producing *E. coli* in Dutch recreational waters influenced by wastewater treatment plants. *Veterinary Microbiology*. 2014;171(3-4):448-459. doi:10.1016/j.vetmic.2014.03.007
16. Jang J, Hur HG, Sadowsky MJ, Byappanahalli MN, Yan T, Ishii S. Environmental *Escherichia coli*: ecology and public health implications—a review. *Journal of Applied Microbiology*. 2017;123(3):570-581. doi:10.1111/jam.13468
17. Fiore E, van Tyne D, Gilmore MS. Pathogenicity of enterococci. *Gram-Positive Pathogens*. 2019;2007(8):378-397. doi:10.1128/9781683670131.ch24
18. Agudelo Higuita NI, Huycke MM. Enterococcal Disease, Epidemiology, and Implications for Treatment. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Published online 2014:1-35.
19. Vila J, Sáez-López E, Johnson JR, et al. *Escherichia coli*: An old friend with new tidings. *FEMS Microbiology Reviews*. 2016;40(4):437-463. doi:10.1093/femsre/fuw005
20. De Oliveira DMP, Forde BM, Kidd TJ, et al. Antimicrobial resistance in ESKAPE pathogens. *Clinical Microbiology Reviews*. 2020;33(3):1-49. doi:10.1128/CMR.00181-19
21. Byappanahalli MN, Nevers MB, Korajkic A, Staley ZR, Harwood VJ. Enterococci in the Environment. *Microbiology and Molecular Biology Reviews*. 2012;76(4):685-706. doi:10.1128/mmbr.00023-12
22. Shrivastava SR, Shrivastava PS, Ramasamy J. World health organization releases global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. *JMS - Journal of Medical Society*. 2018;32(1):76-77. doi:10.4103/jms.jms_25_17
23. Russo TA, Johnson JR. Medical and economic impact of extraintestinal infections due to *Escherichia coli*: Focus on an increasingly important endemic problem. *Microbes and Infection*. 2003;5(5):449-456. doi:10.1016/S1286-4579(03)00049-2
24. World Health Organization. Antimicrobial Resistance in the Western Pacific Region. Published online 2015.
25. Briñas L, Zarazaga M, Sáenz Y, Ruiz-Larrea F, Torres C. β-lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. *Antimicrobial Agents and Chemotherapy*. 2002;46(10):3156-3163. doi:10.1128/AAC.46.10.3156-3163.2002

26. Tendolkar PM, Baghdayan AS, Shankar N. Pathogenic enterococci: New developments in the 21st century. *Cellular and Molecular Life Sciences*. 2003;60(12):2622-2636. doi:10.1007/s00018-003-3138-0
27. Mundy LM, Sahm DF, Gilmore M. Relationships between enterococcal verulence and antimicrobial resistance. *Clinical Microbiology Reviews*. 2000;13(4):513-522. doi:10.1128/CMR.13.4.513-522.2000
28. Richards MJ, Edwards JR, Culver DH, Gaynes RP, Nosocomial N, Surveillance I. N OSOCOMIAL I NFECTIONS IN C OMBINED M EDICAL -S URGICAL I NTENSIVE C ARE U NITS. 2000;21(8).
29. Hollenbeck BL, Rice LB. Intrinsic and acquired resistance mechanisms in *Enterococcus*. *Virulence*. 2012;3(5):421-569. doi:10.4161/viru.21282
30. Afkhami GR. The life and times of the Shah. *The Life and Times of the Shah*. 2009;3(1):46-65. doi:10.1080/00472331003798640
31. Marothi YA, Agnihotri H, Dubey D. Enterococcal resistance - An overview. *Indian Journal of Medical Microbiology*. 2005;23(4):214-219. doi:10.1016/s0255-0857(21)02524-x
32. Palmer KL, Kos VN, Gilmore MS. Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. *Current Opinion in Microbiology*. 2010;13(5):632-639. doi:10.1016/j.mib.2010.08.004
33. Korajkic A, McMinn BR, Staley ZR, Ahmed W, Harwood VJ. Antibiotic-resistant *Enterococcus* species in marine habitats: A review. *Current Opinion in Environmental Science and Health*. 2020;16:92-100. doi:10.1016/j.coesh.2020.07.003
34. Dinos GP. The macrolide antibiotic renaissance. *British Journal of Pharmacology*. 2017;174(18):2967-2983. doi:10.1111/bph.13936
35. Vikesland PJ, Pruden A, Alvarez PJJ, et al. Toward a Comprehensive Strategy to Mitigate Dissemination of Environmental Sources of Antibiotic Resistance. *Environmental Science and Technology*. 2017;51(22):13061-13069. doi:10.1021/acs.est.7b03623
36. McGowan E. Comment on “antibiotic resistance genes as emerging contaminants: Studies in Northern Colorado.” *Environmental Science and Technology*. 2007;41(7):2651-2652. doi:10.1021/es0680156
37. Whitman RL, Harwood VJ, Edge TA, et al. *Microbes in Beach Sands: Integrating Environment, Ecology and Public Health*. Vol 13.; 2014. doi:10.1007/s11157-014-9340-8
38. Zhang Q, Gallard J, Wu B, et al. Synergy between quantitative microbial source tracking (qMST) and quantitative microbial risk assessment (QMRA): A review and prospectus. *Environment International*. 2019;130. doi:10.1016/j.envint.2019.03.051
39. Whitman RL, Harwood VJ, Edge TA, et al. Microbes in beach sands: Integrating environment, ecology and public health. *Reviews in Environmental Science and Biotechnology*. 2014;13(3):329-368. doi:10.1007/s11157-014-9340-8

40. Hancock LE, Murray BE, Sillanpää J. Enterococci: From Commensals to Leading Causes of Drug Resistant Infection. *Enterococcal Cell Wall Components and Structures*. Published online 2014:1-35.
41. Korajkic A, McMinn BR, Harwood VJ. Relationships between microbial indicators and pathogens in recreational water settings. *International Journal of Environmental Research and Public Health*. 2018;15(12). doi:10.3390/ijerph15122842
42. Mudryk ZJ. Antibiotic Resistance among Bacteria Inhabiting Surface and Subsurface Water Layers in Estuarine Lake Gardno. *Polish Journal of Environmental Studies*. 2002;11(4):401-406.
43. Nappier SP, Liguori K, Ichida AM, Stewart JR, Jones KR. Antibiotic resistance in recreational waters: State of the science. *International Journal of Environmental Research and Public Health*. 2020;17(21):1-28. doi:10.3390/ijerph17218034
44. Batt AL, Bruce IB, Aga DS. Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. *Environmental Pollution*. 2006;142(2):295-302. doi:10.1016/j.envpol.2005.10.010
45. Yi X, Lin C, Ong EJL, Wang M, Zhou Z. Occurrence and distribution of trace levels of antibiotics in surface waters and soils driven by non-point source pollution and anthropogenic pressure. *Chemosphere*. 2019;216:213-223. doi:10.1016/j.chemosphere.2018.10.087
46. Rizzo L, Manaia C, Merlin C, et al. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Science of the Total Environment*. 2013;447:345-360. doi:10.1016/j.scitotenv.2013.01.032
47. Łuczkiewicz A, Jankowska K, Fudala-Ksiazek S, Olańczuk-Neyman K. Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. *Water Research*. 2010;44(17):5089-5097. doi:10.1016/j.watres.2010.08.007
48. Alm EW, Burke J, Spain A. Fecal indicator bacteria are abundant in wet sand at freshwater beaches. *Water Research*. 2003;37(16):3978-3982. doi:10.1016/S0043-1354(03)00301-4
49. Whitman RL, Nevers MB. Foreshore sand as a source of *Escherichia coli* in nearshore water of a Lake Michigan Beach. *Applied and Environmental Microbiology*. 2003;69(9):5555-5562. doi:10.1128/AEM.69.9.5555-5562.2003
50. Weiskerger CJ, Brandão J, Ahmed W, et al. Impacts of a changing earth on microbial dynamics and human health risks in the continuum between beach water and sand. *Water Research*. 2019;162:456-470. doi:10.1016/j.watres.2019.07.006
51. Korajkic A, Wanjugi P, Brooks L, Cao Y, Harwood VJ. Persistence and Decay of Fecal Microbiota in Aquatic Habitats. *Microbiology and Molecular Biology Reviews*. 2019;83(4). doi:10.1128/mmbr.00005-19
52. Haack SK, Fogarty LR, Wright C. *Escherichia coli* and enterococci at beaches in the Grand Traverse Bay, Lake Michigan: Sources, characteristics, and environmental

pathways. *Environmental Science and Technology*. 2003;37(15):3275-3282. doi:10.1021/es021062n
53. Mudryk ZJ. Occurrence and distribution antibiotic resistance of heterotrophic bacteria isolated from a marine beach. *Marine Pollution Bulletin*. 2005;50(1):80-86. doi:10.1016/j.marpolbul.2004.10.001
54. Fewtrell L, Kay D. Recreational Water and Infection: A Review of Recent Findings. *Curr Environ Health Rep*. 2015;2(1):85-94. doi:10.1007/s40572-014-0036-6
55. Defeo O, McLachlan A. Patterns, processes and regulatory mechanisms in sandy beach macrofauna: A multi-scale analysis. *Marine Ecology Progress Series*. 2005;295:1-20. doi:10.3354/meps295001
56. Solo-Gabriele HM, Harwood VJ, Kay D, et al. Beach sand and the potential for infectious disease transmission: Observations and recommendations. *Journal of the Marine Biological Association of the United Kingdom*. 2016;96(1):101-120. doi:10.1017/S0025315415000843
57. Heaney CD, Sams E, Dufour AP, et al. Fecal indicators in sand, sand contact, and risk of enteric illness among beachgoers. *Epidemiology*. 2012;23(1):95-106. doi:10.1097/EDE.0b013e31823b504c
58. Bonilla TD, Nowosielski K, Cuvelier M, et al. Prevalence and distribution of fecal indicator organisms in South Florida beach sand and preliminary assessment of health effects associated with beach sand exposure. *Marine Pollution Bulletin*. 2007;54(9):1472-1482. doi:10.1016/j.marpolbul.2007.04.016
59. Heaney CD, Sams E, Wing S, et al. Contact with beach sand among beachgoers and risk of illness. *American Journal of Epidemiology*. 2009;170(2):164-172. doi:10.1093/aje/kwp152
60. Environmental US, Agency P. Water by Membrane Filtration Using Modified membrane-Thermotolerant *Escherichia coli* Agar (Modified mTEC). 2005;(September).
61. (USEPA) USEPA. Enterococci in water by membrane filtration using membrane-*Enterococcus* Indoxyl-β-D-Glucoside Agar (mEI). 2004;(September):2004.
62. Boehm AB, Griffith J, Mcgee C, et al. Faecal indicator bacteria enumeration in beach sand: a comparison study of extraction methods in medium to coarse sands. 2010;107(5):1740-1750. doi:10.1111/j.1365-2672.2009.04440.x.Faecal
63. Gallard-Gongora J, Lobos A, Conrad JW, Peraud J, Harwood VJ. An assessment of three methods for extracting bacterial DNA from beach sand. *Journal of Applied Microbiology*. 2021;(October):1-11. doi:10.1111/jam.15423
64. Anderson KL, Whitlock JE, Valerie J, et al. Persistence and Differential Survival of Fecal Indicator Bacteria in Subtropical Waters and Sediments. *Appl Environ Microbiol*. 2014;68(1):12-23. doi:10.1128/AEM.71.6.3041
65. Chern EC, Brenner KP, Wymer L, Haugland RA. Comparison of Fecal Indicator Bacteria Densities in Marine Recreational Waters by QPCR. *Water Quality, Exposure and Health*. 2009;1(3-4):203-214. doi:10.1007/s12403-009-0019-2
66. Nayak B, Weidhaas J, Harwood VJ. LA35 poultry fecal marker persistence is correlated with that of indicators and pathogens in environmental waters. *Applied*

and Environmental Microbiology. 2015;81(14):4616-4625. doi:10.1128/AEM.00444-15
67. Hudzicki J. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol Author Information. *American Society For Microbiology*. 2012;(December 2009):1-13.
68. Control D, States U. OUTPATIENT ANTIBIOTIC PRESCRIPTIONS — United States , 2015 Healthcare providers prescribed 269 . 4 million antibiotic prescriptions — equivalent to. Published online 2015:1-3.
69. Skarp KP, Shams A, Montelin H, Lagerbäck P, Tängdén T. Synergistic and bactericidal activities of mecillinam, amoxicillin and clavulanic acid combinations against extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* in 24-h time–kill experiments. *International Journal of Antimicrobial Agents*. 2019;53(1):74-79. doi:10.1016/j.ijantimicag.2018.09.011
70. Aguilar-Santelises M, Castillo-Vera J, Gonzalez-Molina R, Garcia del Valle A, Cruz Millan M, Aguilar-Santelises L. Clinical isolates of *Escherichia coli* are resistant both to antibiotics and organotin compounds. *Folia Microbiologica*. 2020;65(1):87-94. doi:10.1007/s12223-019-00707-1
71. Falagas ME, Athanasaki F, Voulgaris GL, Triarides NA, Vardakas KZ. Resistance to fosfomycin: Mechanisms, Frequency and Clinical Consequences. *International Journal of Antimicrobial Agents*. 2019;53(1):22-28. doi:10.1016/j.ijantimicag.2018.09.013
72. Chaves B, Tadi P. Gentamicin. *StatPearls Publishing*. Published online 2022:1-5.
73. Codjoe F, Donkor E. Carbapenem Resistance: A Review. *Medical Sciences*. 2017;6(1):1. doi:10.3390/medsci6010001
74. Dissemond J. Methicillin-resistenter Staphylococcus aureus (MRSA): Diagnostik, klinische Relevanz und Therapie. *JDDG - Journal of the German Society of Dermatology*. 2009;7(6):544-554. doi:10.1111/j.1610-0387.2009.07015.x
75. Davis R, Markham A, Balfour JA. Ciprofloxacin Overview of Antibacterial Activity. Published online 2012:1-139.
76. Bozdogan B, Appelbaum PC. Oxazolidinones: Activity, mode of action, and mechanism of resistance. *International Journal of Antimicrobial Agents*. 2004;23(2):113-119. doi:10.1016/j.ijantimicag.2003.11.003
77. Grossman TH. Tetracycline antibiotics and resistance. *Cold Spring Harbor Perspectives in Medicine*. 2016;6(4). doi:10.1101/cshperspect.a025387
78. Barna C, Williams DH. The Structure and Mode of Action of Glycopeptide Antibiotics of the Vancomycin Group. *Ann Rev Microbiol*. 1984;38(28):339-357.
79. Oates SC, Miller MA, Hardin D, Dominik C, Jessup D, Smith WA. Daily relative dog abundance, fecal density, and loading rates on intensively and minimally managed dog-friendly beaches in central California. *Marine Pollution Bulletin*. 2017;125(1-2):451-458. doi:10.1016/j.marpolbul.2017.10.062
80. Ervin JS, van de Werfhorst LC, Murray JLS, Holden PA. Microbial source tracking in a coastal California watershed reveals canines as controllable sources of fecal contamination. *Environmental Science and Technology*. 2014;48(16):9043-9052. doi:10.1021/es502173s

81. Phillips MC, Solo-Gabriele HM, Piggot AM, Klaus JS, Zhang Y. Relationships between sand and water quality at recreational beaches. *Water Research*. 2011;45(20):6763-6769. doi:10.1016/j.watres.2011.10.028
82. Ahmed W, Zhang Q, Lobos A, et al. Precipitation influences pathogenic bacteria and antibiotic resistance gene abundance in storm drain outfalls in coastal sub-tropical waters. *Environment International*. 2018;116(March):308-318. doi:10.1016/j.envint.2018.04.005
83. Alm EW, Zimbler D, Callahan E, Plomaritis E. Patterns and persistence of antibiotic resistance in faecal indicator bacteria from freshwater recreational beaches. Published online 2014:273-285. doi:10.1111/jam.12512
84. de Oliveira AJFC, Watanabe Pinhata JM. Antimicrobial resistance and species composition of *Enterococcus* spp. isolated from waters and sands of marine recreational beaches in Southeastern Brazil. *Water Research*. 2008;42(8-9):2242-2250. doi:10.1016/j.watres.2007.12.002
85. da Costa Andrade V, Del Busso Zampieri B, Ballesteros ER, Pinto AB, Fernandes Cardoso de Oliveira AJ. Densities and antimicrobial resistance of *Escherichia coli* isolated from marine waters and beach sands. *Environmental Monitoring and Assessment*. 2015;187(6):1-10. doi:10.1007/s10661-015-4573-8
86. Feng F, Goto D, Yan T. Effects of autochthonous microbial community on the die-off of fecal indicators in tropical beach sand. *FEMS Microbiology Ecology*. 2010;74(1):214-225. doi:10.1111/j.1574-6941.2010.00921.x
87. Byappanahalli MN, Shively DA, Nevers MB, Sadowsky MJ, Whitman RL. Growth and survival of *Escherichia coli* and enterococci populations in the macro-alga Cladophora (Chlorophyta). *FEMS Microbiology Ecology*. 2003;46(2):203-211. doi:10.1016/S0168-6496(03)00214-9
88. Skórczewski P, Mudryk Z, Perlin P. Detection of antibiotic resistant bacteria inhabiting the sand of non-recreational marine beach. *Marine Pollution Bulletin*. 2010;60:207-214. doi:10.1016/j.marpolbul.2009.09.025
89. Carney RL, Labbate M, Siboni N, Tagg KA, Mitrovic SM, Seymour JR. Urban beaches are environmental hotspots for antibiotic resistance following rainfall. *Water Research*. 2019;167:115081. doi:10.1016/j.watres.2019.115081

APPENDICES

Appendix A: Environmental Parameters

Table A1. The environmental parameters measured at all three sampling events.

	Courtney Campbell Beach			Ben T. Davis			Cypress Point Park		
	31-Mar-21	13-May-21	14-Jun-21	31-Mar-21	13-May-21	14-Jun-21	31-Mar-21	13-May-21	14-Jun-21
Air temp. (Degrees Celsius)	22	26	27	22	23	26	23	23	26
Humidity	94%	76%	79%	94%	76%	82%	93%	74%	81%
Wind speed (Km/hour)/direction	0	11/NE	6/SSW	0	11/NE	25/SSW	5/N	12/NE	19/SSW
Time	7:30am	7:51am	8:42am	8:18am	8:23am	9:09am	8:52am	8:53am	9:31am
UV	0	1	1	0	1	1	0	1	2
Salinity (ppt)	25	25	27	25	26	28	25.7	26.0	28
Ph	7.97	7.38	7.62	7.94	7.38	7.79	8.03	7.38	7.81
Turbidity	12.5	7.73	4.5	10.9	4.5	9.6	8.6	4.9	8.1
Percent Moisture swash (%)	19.05	19.05	28.95	25	21.95	19.048	21.95	19.05	19.05
Percent Moisture 2.5 meters from swash (%)	16.28	13.64	25	6.38	6.38	4.17	11.11	13.64	2.04
Transect #1	27°57'38.0"N 82°41'58.1"W			27°58'04.3"N 82°34'23.7"W			27°57'0.3"N 82°34'45.6"W		
Transect #2	27°57'38.2"N 82°41'56.5"W			27°58'04.9"N 82°34'24.2"W			27°57'01.2"N 82°32'45.9"W		

Transect #3	27°57'38.3"N 82°41'55.0"W	27°58'05.5"N 82°34'24.7"W	27°57'02"N 82°32'46.4"W

Appendix B: Additional ANOVA Values

Table B1. The additional ANOVA values. DF: degrees of Freedom, SS: sum of squares, F: F-statistic.

ANOVA

Organism	Location								
	Beach			Subsite			Interaction		
	DF	SS	F	DF	SS	F	DF	SS	F
E. coli	2	3.93	2.17	2	1.54	0.85	4	0.96	0.26
Ampicillin-Resistant *E. coli*	2	2.87	4.95	2	2.62	4.52	4	1.2	1.04
Enterococci	2	3.83	4.6	2	5.81	6.98	4	0.41	0.24
Erythromycin-Resistant Enterococci	2	0.92	1.11	2	2.8	3.37	4	2.65	1.6