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Research Article

Evaluation of Monitored and Unmonitored Bacteria in Recreational Beaches in Puerto Rico: Underlining the Environmental Factors that Affect Beach Quality

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Abstract

In Puerto Rico, beaches, specifically *Balnearios*, are a common attraction for people from different parts of the world. For this reason, understanding how the environment can affect the public's health should be a priority. *Vibrio* spp. are gram-negative bacteria naturally found in warm, salty marine environments such as beaches and estuaries. Therefore, this can be a public health safety issue since the beaches are used daily by the public for fishing and recreational activities. The purpose of this study is to (a) to determine if the selected sites comply with the microbial quality standards established by the EPA and EQB of Puerto Rico; (b) to identify and compare the *Vibrio* spp. and Total Coliform concentrations in each sampling site; (c) assess the antibiotic resistance potential of some of the *Vibrio* spp. isolates. Water and sand samples were taken from Escambrón San Juan, Seven Seas Fajardo, Tropical Beach Naguabo, Crash Boat Aguadilla, Punta Salinas Cataño, Playa Rosada Lajas, *Balneario* de Luquillo, *Balneario* de Carolina, and *Balneario* de Rincón [22,23]. For this, we implemented and adapted the EPA 1604 filtration method for the enumeration of Total Coliforms and *Escherichia coli* in water using a 0.45 µm cellulose membrane for both water and sand samples. After the bacteria were isolated, the BiOLOG GEN III system was used to identify through phenotypic colorimetric analysis the bacteria, thereafter, the Kirby Bauer method was used to determine the antibiotic susceptibility patterns [1-6,17-20]. For this test, the following 10 antibiotics were used: Erythromycin, Gentamicin, Doxycycline, Ceftazidime, Sulfa/Trimeth, Ciprofloxacin, Levofloxacin, Ceftriaxone, Tetracycline and Cefotaxime. The result showed that the highest concentrations of bacteria were in Playa Rosada with an average 20,200 CFU/100 mL in mEndo, 10,233 CFU/100 mL in MAC and a 433 CFU/100 mL in TCBS for water and sand samples. The high concentration was due to the accumulation of sargassum along the sand. However, when comparing water and sand samples from all beaches, it was observed that the concentrations were not statistically different P = 0.47 (2 tail). The following three *Vibrio* spp. species were identified in the water and sand samples: *Vibrio alginolyticus*, *Vibrio cholerae* and *Vibrio cholerae non 01* [8]. For antibiotic susceptibility patterns, it was observed that many of the isolates presented resistance to Erythromycin and high susceptibility towards Cefotaxime. In addition, one of the water samples from *Balneario* de Carolina that tested positive for *Vibrio alginolyticus*, demonstrated multiple antibiotic resistance to Erythromycin, Ceftazidime, Sulfa/Trimeth, Tetracycline, and Doxycycline.

Keywords: *Vibrio spp; Beach Quality; BIOLOG; Antibiotic Resistance; Monitored Bacteria; Unmonitored Bacteria*

Abbreviations

EPA: Environmental Protection Agency; EQB: Environmental Quality Board; CDC: Center for Disease Control and Prevention; ESC: Escambrón in San Juan; SVS: Seven Seas in Fajardo; TBN: Tropical Beach in Naguabo; CBA: Crash Boat in Aguadilla; PTS: Punta Salinas in Cataño; PRL: Playa Rosada in Lajas; LUQ: *Balneario* de Luquillo; BCA: *Balneario* de Carolina; BRC: *Balneario* de Rincón; E: Erythromycin; Gm: Gentamicin; D: Doxycycline; CAZ: Ceftazidime; STX: Sulfa/Trimeth; CIP: Ciprofloxacin; LVX: Levofloxacin; CRO: Ceftriaxone; Te: Tetracycline; CTX: Cefotaxime; CFU: Colony Forming Units

Introduction

A Beach is defined as a narrow, gently sloping strip of land composed of non-consolidated soil, also known as sand. They are widely known for being locations were many recreational activities take place. A survey conducted in Puerto Rico in 2013 to 623 people in various beaches determined that the main reason people visited these beaches were: 38% for nature observation, 25% exercise or sports, 17% diving or snorkeling, 7% parties or festivals, 7% conservation activities, 6% Kayak and canoeing, and 5% education and research purposes [7]. Therefore, given that these recreational areas are in constant usage, people who frequent these beaches are unaware of an invisible threat. *Vibrio* spp. are commonly found in these recreational spaces due to their halophilic nature, so it stands to reason that this is a pathogen of concern [9]. However, they are not currently monitored by the Environmental Protection Agency (EPA) or the Environmental Quality Board in Puerto Rico (EQB).

Vibrio (Genus) are group of Gram-negative, curved rod-shaped, halophilic or halotolerant bacteria. Among the pathogenic *Vibrio*, the most virulent are: *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae*. Infection with this bacterium is through the consumption of raw oysters or an open wound exposed to saltwater or brackish water [3-5]. Vibriosis, which can cause nausea, watery diarrhea, skin infections, gastroenteritis, and death if left untreated, can result from exposure. The ideal growth environment for these bacteria is marine-freshwater with a temperature of 20 - 30°C. In many cases, the population prone to vibriosis suffers from liver disease, cancer, HIV, thalassemia, receive immune-suppressing therapy, regularly consume medicine to decrease stomach acid levels, or have had stomach surgery [3]. Vibriosis morbidity rate is at an estimated eighty thousand (80,000) cases yearly [3,4].

Studies have shown that some species of *Vibrio*, such as *Vibrio harveii* and V*ibrio alginolyticus,* are pathogenic to species of seahorses specifically the Yellow seahorse (*Hippocampus kuda*), Leopard whipray (*Himantura uarnak*) and Tahitian stingray (*Himantura fai*), causing skin ulcers and various negative symptoms [24]. This has also impacted marine wildlife such fish, mollusks and other wildlife. Blue crabs (*Callinectes sapidus*) are another marine animal species that are temperature and salinity dependent, which can be affected by *Vibrio* spp.; this is quite worrisome because this species is found on the coast of Puerto Rico. A recent study done in Louisiana showed that the most common species of *Vibrio* found in blue crabs were *Vibrio parahaemolyticus* and *Vibrio cholerae*. The study also showed that the concentrations of bacteria increased as temperatures increased, however, bacterial growth decreased as salinity increased [18].

Climate change can also impact the possible cases of vibriosis we may see at a given time. As climate change warms waterbodies, it makes it more viable for the growth of *Vibrio* [11,13,14]. An example of this is "El Niño", a large-scale ocean-atmosphere climate interaction linked to a periodic warming in sea surface temperatures across the central and east-central Equatorial Pacific [19]. In 2005, due to "El Niño", over 8,000 cases of vibriosis around South America's western coast were reported [16]. "El Niño" phenomenon was at its strongest in 2005, and it affected the water temperature making it preferable for this pathogen. Also, many of the cases were due to ingestion of fish and mollusks infected with *Vibrio parahaemolyticus* and *Vibrio vulnificus* which caused gastroenteritis [16]. Findings like the above mentioned leads us to believe that climate change can be a factor that can affect the cases and the concentrations present of this bacterium.

Now the real question is: Why should this be a concern in Puerto Rico? Given that Puerto Rico is an island with estuarine waters and warm beaches where these bacteria can be prevalent. For this reason, this can be a public health safety issue since the beaches are used daily by the public for fishing and recreational activities. Considering the constant rate of vibriosis infection cases reported across the United States, *Vibrio* spp. should be monitored and regulated in recreational waters [21].

Purpose of the Study

The purpose of this study is to (a) to determine if the selected sites comply with the microbial quality standards established by the EPA and EQB of Puerto Rico; (b) to identify and compare de *Vibrio* spp. and Total Coliform concentrations in each sampling site; (c) assess the antibiotic resistance potential of some of the *Vibrio* spp. isolates.

Materials and Methods

Sampling

The samples were taken from following recreational beaches in Puerto Rico: Escambrón in San Juan (ESC), Seven Seas in Fajardo (SVS), Tropical Beach in Naguabo (TBN), Crash Boat in Aguadilla (CBA), Punta Salinas in Cataño (PTS), Playa Rosada in Lajas (PRL), *Balneario* de Luquillo (LUQ), *Balneario* de Carolina (BCA) and *Balneario* de Rincón (BRC). When first arriving at each sampling site, an observational survey was conducted to determine factors like water temperature and pH, anthropogenic activities, and weather. Each sampling sites was divided into 3 points, and 100 mL of water and approximately 12g of sand were collected per beach, using 100 mL Nasko Whirl-Packs. Samples were placed in a cooler at 4°C upon collection to preserve the environmental conditions they were taken and transported within 4 hours to the laboratory.

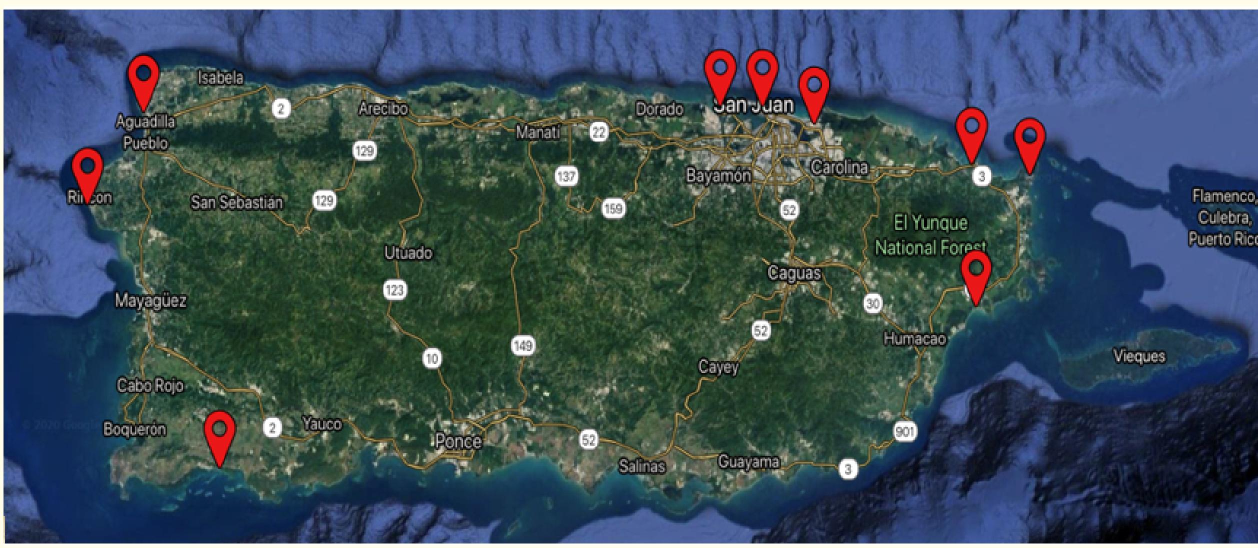


Figure 1: *Specific locations of samples taken in Puerto Rico.*

Processing and filtration

In both the water and sand samples, the filtration method used was the EPA 1604, which is used for the enumeration of Total Coliforms and *Escherichia coli* in water using a 0.45 µm cellulose membrane. For this study's purpose, this method was adapted for the enumeration and detection of Vibrio spp. in water and sand samples. We diluted all the water samples in a 1/100 mL ratio; this implies that 99 mL of distilled water was inoculated with 1 mL of sample water to later determine via statistical analysis the specific concentrations present in 100 mL. The procedure to filtrate and process sand samples included an initial dilution of the 12g of sand samples with 100 mL of distilled water, thereafter, using the same procedure with the water samples applying the 1/100 mL dilution ratio.

Inoculation and incubation

For this study 3 culture media were used, MacConkey agar, a selective and differential culture media that isolates Gram-Negative enteric bacilli, mEndo agar that facilitated the enumeration of coliforms via membrane filtration techniques, and TCBS agar which is selective and differential for the growth of *Vibrio* spp. All culture media were inoculated with the filtered membranes and incubated for 48 hours at 30°C except for the mEndo culture media which was incubated at 35°C.

CFU count and preservation

After 48 hours, the inoculated plates were counted with a manually operated colony counter. During the colony count annotations were taken concerning size, texture, color, and unification. After the colony count, one colony was selected from each TCBS media to be cryogenically preserved for later identification using the BiOLOG ID system and for the determination of antibiotic resistance.

BiOLOG identification process

For the BiOLOG ID, the cryogenically preserved bacteria were transferred to a Brain Heart Infusion (BHI) enriched culture media to activate the bacteria and inoculate the BiOLOG plates. GEN III plates, used for identifying aerobic bacteria and consisting of 71 carbon source assays and 23 chemical sensitivity assays, were used. After the inoculation of the Gen III plates, they were incubated for 18 to 24 hours. After incubation, the plates were read using the BiOLOG MicroStation through phenotypical analysis. The results obtained are the exact or the most probable species of bacteria present in the sample.

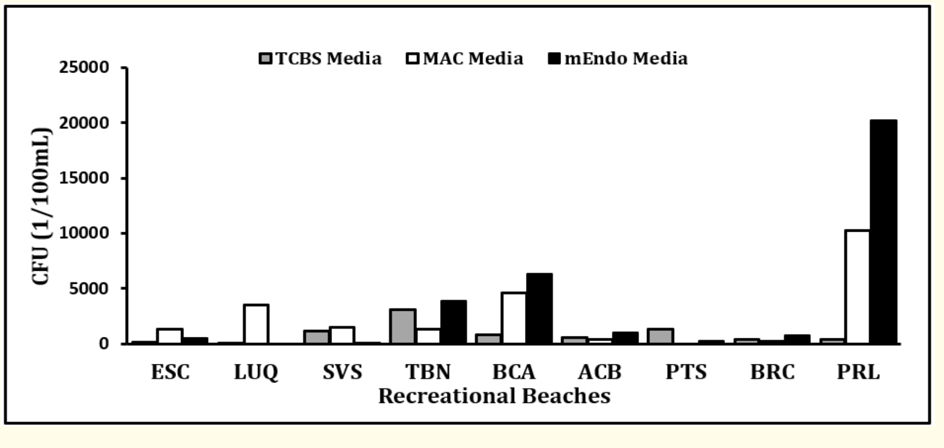
Antibiotic resistance

The method used to assess the antibiotic susceptibility patterns was the Kirby Bauer method. For this study 10 antibiotics were used, these were: Erythromycin (E), Gentamicin (Gm), Doxycycline (D), Ceftazidime (CAZ), Sulfa/Trimeth (STX), Ciprofloxacin (CIP), Levofloxacin (LVX), Ceftriaxone (CRO), Tetracycline (Te) and Cefotaxime (CTX). The reason for choosing those antibiotics is because they are commonly used to treat bacterial infections in hospitals and some are used in the patient treatment of *Vibrio* spp. infections.

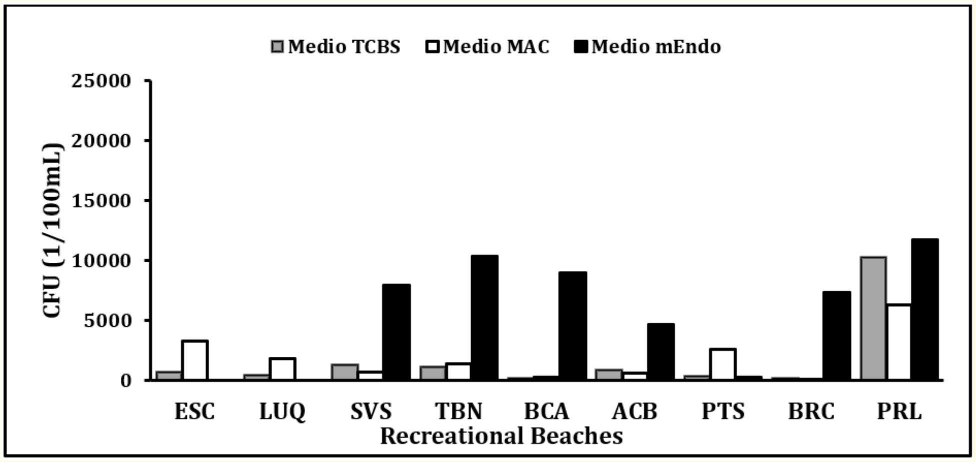
Results and Discussion

Bacterial count analysis

From all sampled beaches, the bacterial concentrations present in water and sand samples were different. The highest bacterial concentrations of water and sand were in Playa Rosada in Lajas, with an average concentration in water samples of 20,200 CFU/100 mL in mEndo, 10,233 CFU/100mL in MAC and a 433 CFU/100 mL in TCBS. These high concentrations of bacteria in both water and sand in Playa Rosada were due to the sargassum, which had the necessary nutrients required for this bacterium to grow. Given that the standards for Total Coliforms is 200 CFU in 100 mL of sample water, these results show that these beaches do not comply with the water quality standards established by the EPA and the EQB. However, when examining all the concentrations of Total Coliform counts in all sampled beaches, the average growth for water is 3688.78 CFU/100 mL and sand being the highest with a 5696.33 CFU/100 mL. Figure 2 demonstrates the average concentrations present in water and sand samples for sampled beaches in TCBS, mEndo and MAC culture media.



(A)



(B)

Figure 2: *These graphs show the different concentrations present in all beaches per culture media, in which (A) is water and (B) is sand.*

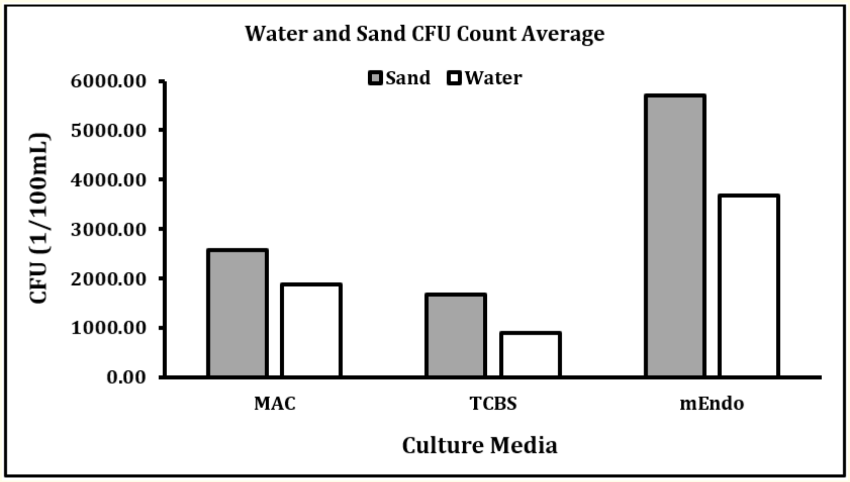
For the MAC culture media, an average of 1,874 CFU/100 mL was observed for water samples and a 2,570/100 mL CFU for sand samples in all the sample sites. In the TCBS culture media, 892 CFU/100 mL was observed for water, and 1,685 CFU/100 mL in the sand was observed for all sample sites. Having these high concentrations of monitored and unmonitored bacteria is a public health issue, especially since there were also high concentrations of bacteria in the sand in most of the samples. This makes the case that monitoring sand should also be important due to possible high counts of coliforms and other enteric bacteria in the sand. An important factor that must be considered is that, in the sand, bacterial concentrations tend to be stagnant and are being exposed to a constant heat source such as the sun, which makes the environment optimal for microbial growth. Therefore, to determine the difference in bacterial presence in the sand and water concentration a T-test: Two Sample for Equal Variances, was performed to the average concentrations for water and sand in all culture media. The results demonstrate that the bacterial concentrations on sand samples (M = 2151.74, SD = 1418.74, n = 3) when compared with the water samples (M = 3317.26, SD = 2107.32, n = 3) resulted in t (4) = 0.79, and P = 0.47 (2 tail), this tells us that these concentrations are not statistically different.

A						
Samples	**Count**	**Mean**	**Standard Deviation**	**Mínimum**	**Median**	**Máximum**
Water Samples	3	3317.26	2107.32	1685.22	2570.22	5696.33
Sand Samples	3	2151.74	1418.74	892.33	1874.11	3688.78

(A)

B			
Samples	**Mean**	**SD**	**P value**
Water Samples	3317.26	2107.32	0.47 NS
Sand samples	2151.74	1418.74	

(B)



(C)

***Figure 3:** This figure demonstrates (A) the descriptive statistics, (B) T-Test: Two Sample Equal Variances and (C) a graph that helps visualize the general concentrations in water and sand samples for all culture media.*

Although our study determined that these concentrations are not statistically different, other studies differ in the water and sand's bacterial concentrations. A study measuring water and sand quality in three beaches off the South Coast, São Paulo State in Brazil, revealed that the concentrations of fecal indicator bacteria (FIB) were higher in dry sand, followed by wet sand and water [2,10,15]. Misic and Anabella [12] stated sandy beaches are considered biofilters since they provide a plethora of ecological services such as the breakdown of organic matter, nutrient mineralization and water filtration. Considering that sand works as a biofilter, it stands to reason that the concentrations of bacteria in the sand will be the same or greater than in water. This further solidifies the necessity for there to be some sand quality parameters.

BiOLOG Identification

Upon identification of isolates, some were false positives due to the lack of a turbidity meter. None the less, a total of 22 isolates were identified as Vibro spp., in which 12 (55%) were from water samples and 10 (45%) were from sand samples. The *Vibrio* species *Vibrio alginolyticus, V. cholerae non 01,* and *V. cholerae* were identified in both water and sand samples. From the water samples, 12 *Vibrio* were identified: 8 were identified as *V. alginolyticus,* 3 were identified as *V. cholerae* non 01, and 1 was identified as *V. cholerae.* From the sand samples, 10 *Vibrio* were identified: 5 were identified as *V. alginolyticus,* 2 were identified as *V. cholerae* non 01, and 3 were identified as *V. cholerae.* These bacterial species are considered part of the beach natural microbial flora, however, many of these species are pathogenic toward humans and marine animals. In addition, many of these bacterial species are linked to coral bleaching, consequently this could lead to ecological and economic impacts. Being that fish inhabit coral reefs, the bleaching effect kills corals with leave fish without a habitat and become vulnerable to other predators, and as a result, a "domino effect" occurs where the fishing industry will i the long run become affected.

	Count (%)	
Species ID	Water	Sand
Vibrio alginolyticus	8 (66%)	5 (50%)
Vibrio cholerae non 01	3 (25%)	2 (20%)
Vibrio cholerae	1 (8%)	3 (30%)

Table 1: *Positively identified Vibrio spp isolated from water and sand samples, in which the total count of identifications was 12 in water and 10 in sand.*

Antibiotic resistance and susceptibility

The Kirby-Bauer method was performed to determine the susceptibility and resistance present in the samples with the previously mentioned antibiotics. In both water and sand samples, the isolates presented an average of 16.43 mm resistance to Erythromycin. The water samples from BCA were positive for *V. alginolyticus* and presented multiple antibiotic resistances in which three of the antibiotics demonstrated an inhibition halo of 0.0 mm. The sample isolated was resistant to Erythromycin, Ceftazidime, Sulfa/Trimeth, Tetracycline and Doxycycline. This is quite worrisome due to this bacterium's prevalence as part of the beach natural microbial flora. Having multiple antibiotic-resistant *V. alginolyticus* poses a public health concern, if bathers were to contract an infection with this bacterium from this beach, the treatment would not be as effective. On the other hand, both water and sand samples presented a high susceptibility average to Cefotaxime.

Antibiotics										
Sample ID	E 15 μg	CRO 30 μg	CIP 5 μg	CAZ 30 μg	SXT 25 μg	Te 30 μg	GM 10 μg	D 30 μg	LVX 5μg	CTX 30 μg
ESC Sand	16.07	19.8	35.22	13.28	29.67	34.42	35.13	36.14	27.4	25.18
SVS Water	21.23	37.81	40.81	37.15	29.29	30.21	23.56	30.17	39.07	39.05
TBN Water	22.51	31.88	30.83	34.59	32.63	31.78	23.07	29.75	35.17	36.4
BCA Water	0.0	17.24	24.92	11.81	0.0	0.0	33.87	11.15	21.72	20.34
TBN Sand	22.76	37.01	37.81	32.36	29.09	32.84	24.57	31.51	36.76	38.2
TBN Sand	17.8	36.2	28.58	35.95	30.42	32.64	20.72	30.93	31.45	38.74
ACB Water	16.73	24.84	17.6	17.72	12.84	20.82	15.66	18.39	16.5	19.58
ACB Water	14.12	27.66	23.47	24.69	22.42	24.935	19.63	23.935	22.295	28.12
ACB Sand	16.61	28.445	24.73	28.1	22.455	23.6	19.54	24.84	22.77	27.26
PRL Water	16.39	26.21	18.97	25.01	18.48	23.13	21.53	23.31	21.33	24.93
PRL Water	16.49	28.39	20.64	25.57	21.6	22.81	16.23	21.14	22.64	28.05

Table 2: *Antibiotic susceptibility and resistance in various samples, the units that were used to measure the inhibition halo were millimeters (mm).*

Conclusion

As previously mentioned, the concentrations of both *Vibrio* spp. and Total Coliforms were higher in Playa Rosada. Upon arriving at Playa Rosada, a large amount of sargassum along the beach's sand was present. This leads us to believe that these high concentrations of bacteria were due to this phenomenon. When examining the compliance with the water quality criteria for Total Coliforms in all the water samples, we can observe that the sample sites do not comply with EPA standards. As for sand samples, the Total Coliform concentrations present were as high as 5696.33 CFU, this could be due to the heat capacity, the stability and the nutrient content of the sand. This implies that sand can absorb more heat, offer a stable surface and more nutrients than water, making the environment optimal for bacterial growth. When comparing these concentrations with water, they were not statistically different P = 0.47 (2 tail). However, many studies conclude that the sand samples' concentrations tend to be higher than in water. For this reason, it is recommended that some parameters should be established for sand samples, and of course, shouldn't be limited to regulated bacteria but should include other microorganisms such as unregulated bacteria, fungi, viruses, and parasites. When using the EPA 1604 method adapted for the enumeration of *Vibrio* spp. and the usage of this method to determine the concentrations in the sand, we found that it was more efficient, cost-effective and less time-consuming.

For identification purposes, using the BiOLOG ID was very efficient; however, we recommend using a turbidity meter to limit the probability of false-positive identifications. The bacteria identified in both water and sand samples were *V. alginolyticus*, *V. cholerae,* and *V. cholerae* non O1. As seen in the literature, many of the identified bacteria are linked to gastrointestinal infections, open wound infections, marine animal infections such as mollusks and fish, and the coral bleaching effect in the ocean. Many of the isolates presented resistance to Erythromycin and high susceptibility towards Cefotaxime. In addition, one of the water samples from the *Balneario* de Carolina (BCA) was positive for *V. alginolyticus*, demonstrating multiple antibiotic resistance to Erythromycin, Ceftazidime, Sulfa/Trimeth, Tetracycline and Doxycycline. This present an additional concern. Many pathogenic bacteria are not monitored or are unregulated. These bacteria could present strong antibiotic resistance thus becoming a public health concern for those people that could be exposed and infected by those bacteria.

It is important that the existing laws and regulations are reviewed, as well as that new laws and regulations, that are guided by the reality of the scientific data, are created. This with the objective of protecting and ensuring the health and safety of our environment and community on the beaches and *Balnearios*.

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Conflict of Interest

We do not declare any conflict of interest.

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