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Article

Enriched Abundance of Diverse *Vibrio parahaemolyticus* in the Dead-End of the Ala Wai Canal: A Tropical Artificial Urban Estuary

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Cite This: https://doi.org/10.1021/acsestwater.4c00368

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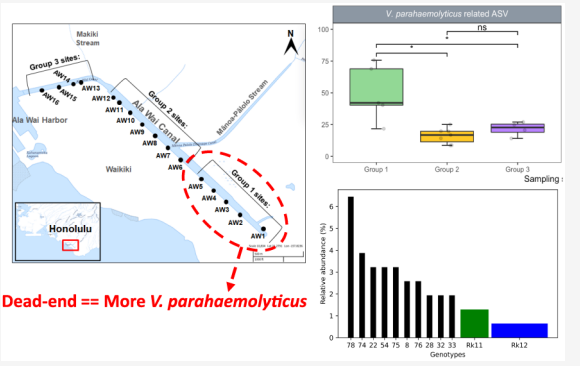
ABSTRACT: *Vibrio* pathogens in marine estuaries pose significant human health risks through recreational water use and seafood contamination. This study used the Ala Wai Canal in Honolulu, Hawaii, as a model tropical urban marine estuary to understand the *Vibrio* population's composition and associated environmental health risks. *Vibrio*-specific 16S rRNA gene amplicon sequencing identified diverse *Vibrio* populations. Among the 62 major amplicon sequencing variants (ASVs) detected, *V. parahaemolyticus*-clustered ASVs were significantly more prevalent than those clustered with *V. cholerae* and *V. vulnificus*. Spatial distribution of the *Vibrio* ASVs showed that *V. parahaemolyticus*-clustered ASVs were highly abundant toward the stagnant end of the canal with higher turbidity and BOD_5 than sites near the freshwater inlets and the ocean outlet. DNA fingerprinting of *V. parahaemolyticus* isolates obtained from the water showed significant genetic diversity, corresponding to the ASV-based diversity and indicating the presence of diverse environmental niches. The *V. parahaemolyticus* population in the canal water exhibited a low detection frequency of the virulence factor *tdh* and an antibiotic resistance profile typical of environmental isolates. The identification of *V. parahaemolyticus* as the most prevalent *Vibrio* pathogen and its enrichment within the stagnant terminus of the canal suggest that increased water circulation may change *Vibrio* ecology and alleviate potential health risks.



KEYWORDS: *amplicon sequencing variants (ASV), genetic diversity, Vibrio parahaemolyticus, rep-PCR, tropical estuary*

1. INTRODUCTION

The Ala Wai Canal is an artificial tropical urban estuary in Honolulu, Hawaii, which was constructed in the 1920s to drain the wetlands on Oahu's south shore and is now used for flood control and for recreational purposes. Because of storm and urban drainage, as well as occasional sanitary sewer overflow (SSO) events from surrounding urban watersheds, water quality has been a persistent concern. In 2006, a large SSO event led to a major fecal contamination in the canal, and around the same time, an unfortunate fatal case of *V. vulnificus* infection occurred in an individual who fell into the Ala Wai Yacht Harbor, which is located at the ocean end of the canal.[1] This has brought attention to the risk of *V. vulnificus* in and around the canal, and several studies have been performed to monitor and predict the abundance of *V. vulnificus* in the canal water.[2,3] However, there remains a major knowledge gap in the prevalence, diversity, and health risks of other *Vibrio* pathogens in the Ala Wai Canal.

The most common *Vibrio* pathogens include *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus*.[4] Although *V. cholerae* remains a significant human health threat and causes about 1.3 to 4.0 million cases each year globally,[5] vibriosis caused by noncholera *Vibrio* is more prevalent in the U.S., accounting for approximately 80 000 illnesses and 100 deaths each year.[6] The common routes of vibriosis infections include oral uptake through consumption of raw, undercooked, or contaminated seafood, and direct contact with contaminated seawater.[7,8] Among the noncholera *Vibrio*, *V. parahaemolyticus* is the most prominent species causing approximately 45 000 illnesses.[6]

The Ala Wai Canal represents a unique engineered system to understand *Vibrio* ecology and health risks in tropical marine estuary environments that are impacted by urban environments. First of all, the year-round warmer tropical coastal water of the Hawaii islands, with an average yearly temperature range of 22−26 °C,[9] is expected to favor *Vibrio* ecology. This is corroborated by the high incidence rate of noncholera *Vibrio* infections in Hawaii.[6] Studies in other regions have shown that the *Vibrio* population tends to remain consistent over the year in tropical and subtropical waters[10] and more frequently detected in summer months than in

Received: April 28, 2024
Revised: August 1, 2024
Accepted: August 1, 2024

winter months in temperate regions.[11−15] Second, the constant freshwater input and nutrient and sediment loading from the surrounding urban watersheds also provide conditions that may be favorable for *Vibrio* growth. Studies in other areas have shown that the population composition and dynamics of *Vibrio* in aquatic environments are affected by various environmental conditions, including salinity, nutrients, and sediments.[16−18] Furthermore, the engineering design of the Ala Wai Canal, including the presence of a dead-end terminus and multiple freshwater input points, creates considerable spatial gradient and temporal dynamics of temperature, salinity, nutrient, and sediment, which are expected to affect *Vibrio* growth and distribution in the canal.

The aim of this study was to characterize the microbiological risk imposed by the *Vibrio* population in Ala Wai water with respect to the canal configuration. Amplicon sequencing with *Vibrio*-specific primers was first conducted to characterize the overall *Vibrio* diversity along the canal. The relative abundance of sequences clustered with the common *Vibrio* pathogens (i.e., *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*) was determined and analyzed with respect to sampling locations and environmental parameters. Since *V. parahaemolyticus* was detected as the most prevalent *Vibrio*, *V. parahaemolyticus* isolates were obtained and subjected to rep-PCR DNA fingerprinting in order to characterize the genetic diversity. Finally, multiplex PCR and broth microdilution analyses were conducted on the *V. parahaemolyticus* isolates to determine the virulence factor profile and antimicrobial resistance patterns, respectively.

2. MATERIALS AND METHODS

2.1. Sample Collection and Environmental Parameter Measurements. Water samples were collected from 16 sites (AW1−AW16) along the Ala Wai Canal, which is a 3.1-km long engineered urban marine estuary on the island of Oahu, Hawaii (Figure 1). The sampling locations were divided into 3 groups based on their locations with respect to freshwater input points and the ocean. Group 1 sites (AW1−AW5) were located at the dead-end of the Ala Wai Canal with occasional input of surface water runoffs from surrounding urban areas. Group 2 sites (AW6−AW12) were located immediately downstream of the inlet of the Mānoa−Pālolo Stream, the biggest and constant freshwater input that contains runoff from upstream urban and forest areas. Group 3 sites (AW13−AW16) were located immediately downstream of the inlet of the Makiki Stream, which also contains runoff from upstream urban and forest areas, and closer to the Ala Wai Harbor.

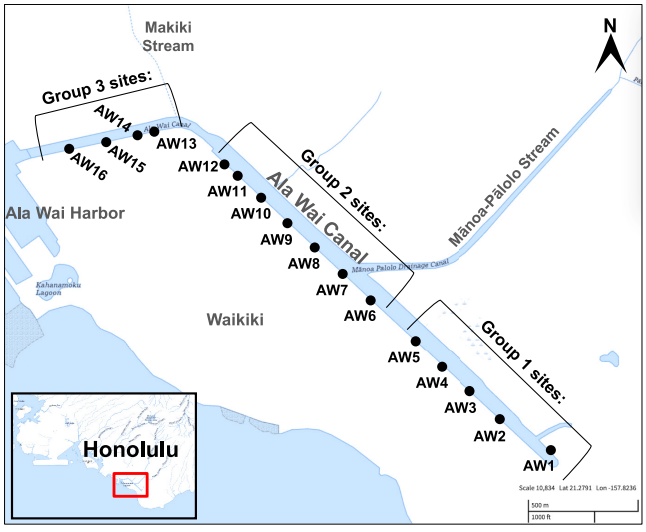


Figure 1. Location of 16 sampling sites (AW1−AW16) along the Ala Wai Canal on the Island of Oahu, Hawaii. Group 1 sampling sites (AW1−AW5) are located at the dead-end of the Ala Wai Canal with occasional input of surface water runoffs; group 2 sites (AW6−AW12) are located after the inlet of the Mānoa−Pālolo Stream; and group 3 sites (AW13−AW16) are located after the inlet of the Makiki Stream and closer to the Ala Wai Harbor.

Samples were collected from the water surface near the canal bank in sterilized polypropylene bottles. During water sampling, temperature, pH, and dissolved oxygen (DO) were measured using a YSI ProDSS Multiparameter Digital Water Quality Meter (YSI, Xylem Inc.; Yellow Springs, OH, USA), and salinity was measured using a Salinity Pocket Tester (VWR; West Chester, PA, USA). All samples were stored on ice and transported to the laboratory for immediate processing (within 6 h). In the laboratory, total suspended solids (TSSs) and 5-day biological oxygen demand (BOD_5) were measured following the standard method 2540D and 5210B, respectively.[19] Results are shown in Figure S1.

2.2. *Vibrio*-Specific 16S rRNA Gene Amplicon Sequencing and Bioinformatic Analysis. To understand the *Vibrio* population composition and diversity, water samples (1 L) were first filtered through 0.45 μm-pore membrane filters, and the membrane filters were cut into small pieces and used for total genomic DNA extractions using a PowerSoil DNA Extraction Kit (Qiagen; CA, USA) by following the manufacturer's protocol. The extracted DNA samples were stored at −80 °C until further analyses. The 16S rRNA gene was amplified using the *Vibrio*-specific primers, VF169 forward (5′-GGATAACYATTGGAAACGATG-3′) and Vib-680R reverse (5′-GAGACAGGAAATTCTACCCCCCTCTACAG-3′).[20] The 20 μL PCR reaction mixture contained a 1× GoTaq qPCR master mix, 0.5 μM forward and reverse primers each, and 0.16 mg/mL BSA. The thermocycling conditions include an initial 95 °C for 2 min followed by 30 cycles of 95 °C for 15 s, 52 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 7 min. Amplification was confirmed by the expected band size of the PCR amplicons (534 bp) through agarose gel electrophoresis. Library preparation, multiplex indexing, and subsequent sequencing on an Illumina Miseq system was performed by the DNA service facility at the University of Illinois at Chicago.

An average of 12 181 paired-end sequencing reads with the length of 301 bp for each pair end were generated for each sample. Paired-end sequence reads were quality trimmed and denoised, and amplicon sequencing variants (ASVs) were picked using the denoise-single command in DADA2 package[21] in QIIME2[22] with default parameters. Taxonomic classification of ASVs was performed by using QIIME2 and Silva 132 (99% database)[23] with default parameters, which assigned all ASVs to the *Vibrio* genus (Table S1). To further analyze the diversity of the *Vibrio* population at the species level, ASVs were aligned to the expected amplicons of the 16S rRNA gene from known *Vibrio* species. The 16S rRNA fragments of known *Vibrio* spp. (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. mimicus*, *V. fluvialis*, and *V. furnissii*) were retrieved from the reference genomes. The phylogenetic tree (Figure 2) was constructed through the maximum likelihood method and Tamura−Nei

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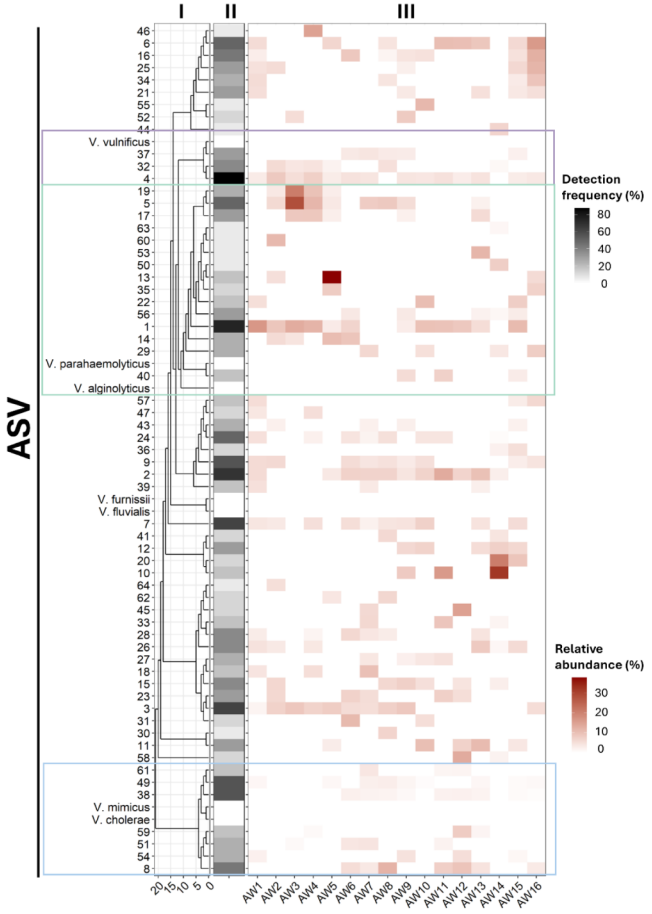

Figure 2. Phylogenetic relationship of the 62 *Vibrio* ASVs from the Ala Wai Canal water with respect to 7 common human *Vibrio* pathogens (*V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus*, *V. furnissii*, and *V. fluvialis*). The detection frequency (%) of each ASV in the 16 samples is shown in the gray-scaled heatmap, while the relative abundance (%) of the ASVs at each sampling site is shown in the red-scaled heatmap. The colored boxes indicate ASVs affiliated with *V. vulnificus* (purple), *V. parahaemolyticus* and *V. alginolyticus* (green), and *V. cholerae* and *V. mimicus* (blue).

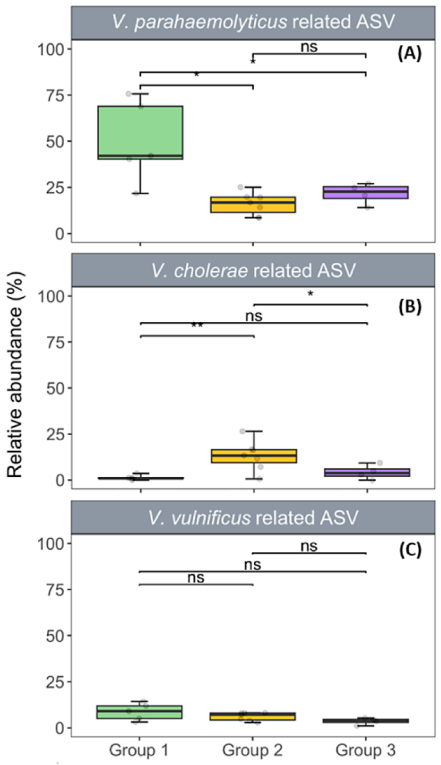

Figure 3. Relative abundance of *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* clustered ASVs between sampling sites of groups 1, 2, and 3 in the Ala Wai Canal. Significance differences between groups were marked by asterisks: * represents $p < 0.05$ and ** represents $p < 0.01$. No significance between groups was shown as "ns".

model in MEGA X.[24] The relative abundances of *Vibrio* species-clustered ASVs in each sampling group were compared (Figure 3). Statistical analyses and visualizations were conducted with R (version 4.2.1) using vegan and ggplot2.

2.3. *Vibrio parahaemolyticus* Isolation. A total of 155 *V. parahaemolyticus* isolates obtained from the Ala Wai Canal from a previous study[25] were used for genetic diversity analysis in this study. The sampling site names from the previous study (sites 1−5) were equivalent to the sampling site names (AW1, AW5, AW14, AW12, and AW9, respectively) in this study. Briefly, *Vibrio*-positive enrichment cultures were streaked on CHROMagar *Vibrio* agar plates (CHROMagar; Paris, France) and incubated at 37 °C for 24 h following manufacturer's instruction. Presumptive *Vibrio* colonies were picked and streaked again on CHROMagar *Vibrio* agar plates for purification. The putative *Vibrio* isolates were further tested of their growth in Tryptone Salt Broth with 3% NaCl (T_0N_3 broth) or without additional salt (T_0N_0 broth).[26] The isolates with growth only in T_0N_3 broth were confirmed as *V. parahaemolyticus*. Phenotypically verified *V. parahaemolyticus* isolates (n = 155) were cultured in APW broth and stored at −80 °C with 16% glycerol for further analyses.

Nine of the phenotypically verified *V. parahaemolyticus* isolates were randomly picked for 16S rRNA gene amplification and sequencing by using the universal 16S rRNA gene primers 27F and 1492R. The sequencing reaction was performed using the ABI 3730XL sequencer at the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB), University of Hawaii at Manoa. The sequence reads were quality trimmed and checked manually against the chromatograms by using a Sequence Scanner (ver. 2.0; Thermo Fisher, Waltham, MA, USA), and the species identification was confirmed by using BLASTn. The nine 16S rRNA gene sequences were deposited in the GenBank database (accession nos. ON965235−ON965243).

2.4. DNA Fingerprinting by Rep-PCR. DNA fingerprinting of the Ala Wai Canal *V. parahaemolyticus* isolates (n = 155) was performed by following a procedure modified from a previous study.[27] Total genomic DNA of *V. parahaemolyticus* isolates was extracted by freeze-and-thaw treatment.[28] *V. parahaemolyticus* was cultured in TSB supplemented with 2% NaCl culture at 35 °C for 24 h, and 10-fold dilution of the culture in sterilized deionized water was subjected to freeze-and-thaw treatment at −20 °C for 24 h. The processed diluted cultures with extracted DNA were directly used for rep-PCR DNA fingerprinting. The rep-PCR reaction mixture contained 0.16 mg/mL BSA, 1% Triton X-100, 200 μM dNTPs, 0.08 U/μL Taq polymerase, and 4.14 μM primer in a final volume of 12 μL. The thermocycling program included initial denaturation at 95 °C for 7 min, followed by 35 cycles of 90 °C for 30 s, annealing at 45 °C for 1 min, extension at 72 °C for 5 min, and a final extension at 70 °C for 10 min. PCR products were

https://doi.org/10.1021/acsestwater.4c00368
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separated by gel electrophoresis using 1.5% agarose at 100 V for 30 min followed by 75 V for 4 h. Gel with PCR amplicons was stained in 1× GelRed solution (Biotium; Hayward, CA) and visualized using a GelDoc imager (Bio-Rad, Hercules, CA).

DNA fingerprints were analyzed using BioNumerics v.7.01 (Applied Maths Inc., Sint-Martens-Latem, Belgium). The level of similarity between fingerprints was calculated using the Dice coefficient at 1.0% optimization and 1.0% band position tolerance. Bands up to 3 kbp were included in the analysis. The between-gel variation between the replicate runs of the positive control *V. parahaemolyticus* clinical strain was used to determine the cutoff threshold in grouping the isolates into different genotypes. *V. parahaemolyticus* positive controls were clustered at 84.6% similarity, which was then used as a cutoff value for genotype assignment.

2.5. Antibiotics Susceptibility Testing. Antimicrobial susceptibility of the *V. parahaemolyticus* isolates (n = 155) was determined by using the broth microdilution method.[29] In brief, *V. parahaemolyticus* isolates were streaked on tryptic soy agar (TSA) supplemented with 2% NaCl, and a fresh single colony was cultured in the cation-adjusted Mueller Hinton Broth (CAMHB) at 35 °C until the cell turbidity reached the equivalent of 0.5 McFarland standard (~5 × 10^8/mL). Approximately 5 × 10^5 cells were inoculated to a 96-well cell culture plate of CAMHB supplemented with antibiotics. The antibiotics tested included ampicillin (1−256 μg/mL), cefotaxime (0.25−8 μg/mL), cefoxitin (1−64 μg/mL), ceftazidime (0.5−32 μg/mL), meropenem (0.25−8 μg/mL), amikacin (2−256 μg/mL), gentamicin (0.5−32 μg/mL), tetracycline (0.5−32 μg/mL), ciprofloxacin (0.25−8 μg/mL), chloramphenicol (1−64 μg/mL), and cefazolin (0.5−32 μg/mL). The broth microdilution plates were incubated at 35 °C for 20 h. The minimal inhibitory concentration (MIC) was recorded and compared with published resistance breakpoints to determine antibiotic resistance.[30] Undefined MIC was substituted by a double value of the highest tested concentration. *Escherichia coli* ATCC 25922 was used to quality test the media with supplemented antibiotics.

2.6. Detection of Virulence Genes. Detection of *tlh*, *tdh*, and *trh* in the *V. parahaemolyticus* isolates (n = 155) was performed using a multiplex PCR assay.[31] The details of the primers used were summarized in Table S3. The 12 μL reaction mixture contained 0.16 mg/mL BSA, 200 μM dNTPs, 0.025 U/μL Taq polymerase, 0.1 μM *tlh* forward and reverse primers, 0.5 μM *tdh* and *trh* forward and reverse primers, and 1 μL DNA template. The thermocycling program included an initial denaturation at 94 °C for 3 min, 25 cycles of denaturation (94 °C for 1 min), annealing (60 °C for 1 min), extension (72 °C for 2 min), and a final extension (72 °C for 3 min). PCR amplicons were separated by gel electrophoresis in a 3.0% agarose gel. Gel with PCR amplicons was stained in 1× GelRed solution (Biotium; Hayward, CA) and visualized using a GelDoc imager (Bio-Rad; Hercules, CA). A laboratory *V. parahaemolyticus* strain that carries the *tlh*, *tdh*, and *trh* genes was used as the positive control for the multiplex PCR assay.

2.7. Statistical Analysis. The environmental parameters and ASV data were statistically analyzed by conducting Pearson's parametric correlation analysis to determine the significant relationship between the environmental parameters, ASV count numbers, and ASV total sequence reads by each sampling site (Figure S2). To better understand the variations and correlation of the ASV community structure with sampling sites and the environmental parameters, detrended correspondence analysis (DCA) was used prior in order to give a decision to select either a unimodal or linear ordination model.[32] Due to the value of the length of gradient result via DCA, canonical correspondence analysis (CCA) was selected to perform a triplot of clustering sampling sites and ASV data with environmental parameters as vectors. The result from CCA was further examined by ANOVA-like permutation tests for significance of constraints to identify patterns of ASV detection, in particular sampling sites that have a relationship with the environmental variables. All statistical analyses and visualizations were conducted with R (version 4.2.1) using the ggiNEXT, ggcorrplot, and PerformanceAnalytics packages.

3. RESULTS

3.1. Prevalence and Diversity *Vibrio* Population. The *Vibrio* population diversity in the Ala Wai Canal was assessed through amplicon sequencing with *Vibrio*-specific 16S rRNA gene primers. A total of 321 ASVs of the *Vibrio* genus-specific 16S rRNA gene fragment were detected in the 16 water samples, and 62 major ASVs (i.e., ≥ 5% relative abundance in at least one sample) were clustered according to their phylogenetic relationship with the common *Vibrio* pathogens (Figure 2). Among the ASV clusters related to *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, the *V. parahaemolyticus* contained 15 different ASVs, higher than the *V. cholerae* cluster (7 ASVs) and the *V. vulnificus* cluster (3 ASVs).

The relative abundances of the three main ASV groups that are clustered with *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* were further analyzed to determine if their detection differs based on the grouping of site locations along the Ala Wai Canal (i.e., groups 1−3) (Figure 3). *V. parahaemolyticus*-clustered ASVs (Figure 3A) exhibited the highest relative abundances across all sampling sites when compared to *V. cholerae*- and *V. vulnificus*-clustered ASVs. In particular, the group 1 sites near the dead-end of the canal had a significantly higher relative abundance of *V. parahaemolyticus*-clustered ASVs (49.7%) than those in group 2 sites (16.1%; p = 0.026) and those in group 3 sites (21.6%; p = 0.045). Group 2 and group 3 sites showed no significant difference (p = 0.18).

The other two ASV clusters showed different patterns with respect to the sampling site locations (Figure 3BC). The *V. cholerae*-clustered ASVs in group 2 sites (13.2%) were significantly higher than those in group 1 sites (1.3%; p = 0.0079) and group 3 sites (4.3%; p = 0.037); whereas group 1 and group 3 sites showed no significant difference (p = 0.23). *V. vulnificus*-clustered ASVs showed no significant difference (p > 0.05) between the groups of sites.

3.2. Genotypic Diversity of *V. parahaemolyticus*. Since *V. parahaemolyticus*-clustered ASVs were the most prevalent when compared with the other *Vibrio* spp., *V. parahaemolyticus* isolates obtained from the Ala Wai canal were characterized by DNA fingerprinting to determine the genetic diversity of *V. parahaemolyticus* species in the water body. Out of the 155 isolates characterized, a total of 93 *V. parahaemolyticus* genotypes were identified from rep-PCR DNA fingerprinting analysis. The *V. parahaemolyticus* genotypic richness, species evenness, and species diversity in the Ala Wai Canal were shown in Figure 4. Rank abundance analysis showed that *V. parahaemolyticus* isolates in the Ala Wai Canal have great richness (93 genotypes were ranked in total) but low evenness (a steep gradient observed at the high-ranking genotypes,

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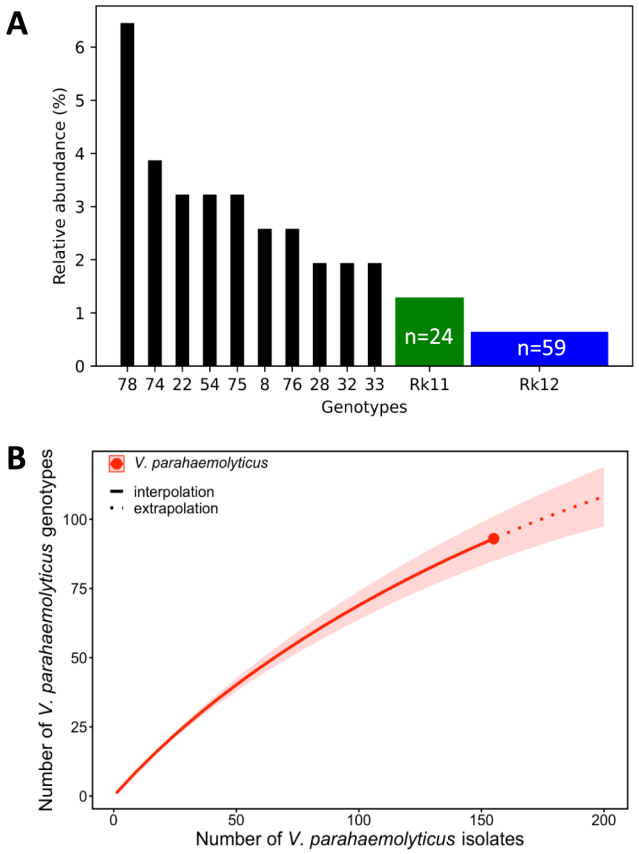

Figure 4. Rank abundance curve (A) and rarefaction curve (B) showed the *V. parahaemolyticus* genotypic richness and species evenness in the Ala Wai Canal. In Figure 4A, 10 *V. parahaemolyticus* genotypes have more than two genetically identical isolates (black bars), 24 *V. parahaemolyticus* genotypes have two genetically identical isolates (green bar), and 59 *V. parahaemolyticus* genotypes have a single isolate (blue bar). Rk indicates the rank. The list of genotypes in Rk 11 and Rk 12 is summarized in Table S6. In Figure 4B, the solid line (interpolation) represents the actual diversity counts and the dashed line (extrapolation) shows the predicted diversity. The shaded region is the 95% confidence level.

which have much higher abundances than the low-ranking genotypes) (Figure 4A). Among the genotypes with higher abundance, 10 genotypes (genotype numbers: 78, 74, 22, 54, 75, 8, 76, 28, 32, and 33; black bars) have a range of 3 to 10 isolates in each genotype. The low-ranking genotypes, i.e., genotypes that have only 1 or 2 *V. parahaemolyticus* isolates, were shown in aggregated ranks (i.e., rank 11 and rank 12). Twenty-four genotypes in rank 11 have two identical isolates, while fifty-nine genotypes in rank 12 contain only a single isolate. The list of the genotypes in rank 11 and rank 12 is summarized in Table S2. The rarefaction curve not approaching a plateau (Figure 4B) indicated that the number of isolates obtained has not exhausted the *V. parahaemolyticus* diversity in the water body. Together, these data demonstrated tremendous genetic diversity among the *V. parahaemolyticus* isolates from the Ala Wai Canal water.

3.3. Antibiotic-Resistant Pattern and Virulence Factor Profile of *V. parahaemolyticus* Isolates. Antibiotic resistances (AR) of the 155 *V. parahaemolyticus* isolates from the Ala Wai Canal were shown in Figure 5. Most of the *V. parahaemolyticus* isolates (average 81.9%) from all five sampling sites showed antibiotic resistance to cefazolin−

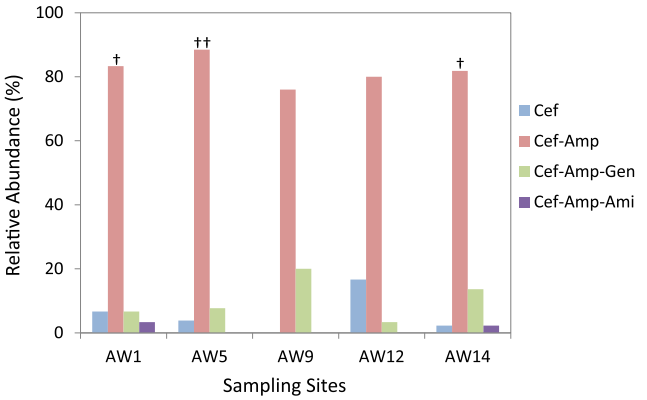

Figure 5. Antibiotic-resistant profile of 155 *V. parahaemolyticus* isolates isolated from the Ala Wai Canal. The highest antibiotic-resistant profile possessed by *V. parahaemolyticus* was Cef−Amp (average 81.9%), followed by Cef−Amp−Gen (average 10.3%), Cef (average 5.9%), and Cef−Amp−Ami (average 1.1%) (Cef: cefazolin; Amp: ampicillin; Gen: gentamicin; and Ami: amikacin). Four *V. parahaemolyticus* isolates possessing a virulence gene *tdh* were marked with the dagger symbol (†) according to their antibiotic-resistant profile and sampling sites.

ampicillin, followed by cefazolin−ampicillin−gentamicin (average 10.3%), cefazolin (average 5.9%), and cefazolin−ampicillin−amikacin (average 1.1%). One *V. parahaemolyticus* isolate showed no antibiotic resistance to any antibiotic tested. *V. parahaemolyticus* isolates from the Ala Wai Canal were not resistant to cefotaxime, cefoxitin, ceftazidime, chloramphenicol, ciprofloxacin, meropenem, and tetracycline (data not shown). All isolates were positive of the *V. parahaemolyticus* marker gene *tlh*, while no isolates were positive of the *trh* gene and only four isolates (2.6%) were positive of the *tdh* gene. All 4 *tdh*-positive isolates exhibited the same AR profile of antibiotic resistance to cefazolin−ampicillin.

4. DISCUSSION

In this study, the ASVs that are closely clustered with *V. parahaemolyticus* were more abundant than those ASVs clustered with *V. cholerae* and *V. vulnificus*, regardless of the locations along the Ala Wai Canal. This observation is in agreement with a previous study where efforts to isolate *Vibrio* resulted in *V. parahaemolyticus* isolates almost exclusively.[25] This apparent prevalence of *V. parahaemolyticus*, based on both the cultivation-independent amplicon sequencing in this study and previous cultivation efforts,[25] may be attributed to *V. parahaemolyticus*'s broad tolerance of temperature (e.g., growth temperature as low as ≤10 °C and typically ≥20 °C[33,34]) and salinity (e.g., 20−30 ppt[34,35]). The presence of a significant amount of sediment in the shallow canal water is another potential factor. The Ala Wai Canal is a type B estuary with an estimated tidal prism volume (V_p) 16 times of the freshwater input (V_f) and limited sediment export to the ocean.[36] Previous studies often reported *V. parahaemolyticus* being the most frequently detected among the three *Vibrio* pathogens in coastal sediments of the Mediterranean Sea.[18]

The spatial distribution patterns of *V. parahaemolyticus*-clustered ASVs along the Ala Wai canal appeared to coincide with the physical configuration of the canal and hydrodynamic conditions created by the major freshwater inlets and ocean outlet. The highest abundance of *V. parahaemolyticus*-clustered ASVs was detected toward the dead-end terminus of the canal,

https://doi.org/10.1021/acsestwater.4c00368
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while its relative abundance is much smaller toward the inlet draining of the two fresh streams and toward the ocean. *V. parahaemolyticus*-clustered ASVs accounted for 49.7% of total ASVs in the group 1 sites, 16.1% in the group 2 sites, and 21.6% in group 3 sites (Figure 3A). Similar spatial distribution, albeit at a much reduced magnitude, was also observed of the *V. vulnificus*-clustered ASVs (Figure 3C), which is in agreement with a previous study showing similar spatial variability of the *V. vulnificus*-specific hemolysin gene marker *vvhA* abundance in the Ala Wai Canal.[2]

The accumulation of *V. parahaemolyticus*-clustered ASVs toward the dead-end terminus of the Ala Wai canal is probably caused by the higher turbidity and nutrient availability in the water and underlying sediment layer. The tributary streams routinely introduce suspended solids and organic and inorganic nutrients from the surrounding urban areas to the Ala Wai Canal. The group 1 sites tend to have higher TSS and BOD_5 than the other sites (Figure S1), and correlation analysis showed a positive correlation between ASVs in the group 1 sites and both TSS and BOD_5 of the water samples (Figure S2). Previous studies have shown that a higher number of *V. parahaemolyticus* was often positively correlated with higher turbidity.[35]

To assess the microbiological risks posed by the abundant *V. parahaemolyticus* in the Ala Wai Canal water, *V. parahaemolyticus* isolates were obtained and its genetic diversity was analyzed. The large number of ASVs clustered with *V. parahaemolyticus* (Figure 2) also corroborates with the high diversity observed with the *V. parahaemolyticus* isolates from the Ala Wai Canal (Figure 4). Previous studies on *V. parahaemolyticus* of environmental sources have generally shown high genetical heterogeneity.[37,38] For example, a total of 167 clinical and environmental *V. parahaemolyticus* isolates from the Pacific Northwest Coast of the U.S. were separated into the 39 rep-PCR group, in addition to the 24 multilocus sequence type (MLST) suggesting that this species is polyphyletic in nature.[39] The diversity of *V. parahaemolyticus* in the tropical marine estuary usually fluctuates based on environmental parameters such as temperature, salinity, suspended solids, nutrients, and zooplankton communities.[11,40]

Fortunately, the highly abundant and diverse *V. parahaemolyticus* population in the Ala Wai Canal does not appear to possess an unusually high microbiological risk based on the characterization of and virulence factors and antibiotic resistance pattern (Figure 5). In this study, only 2.6% of the *V. parahaemolyticus* isolates from estuarine water were found to possess the *tdh* gene, and no *trh* gene was detected. Most environmental *V. parahaemolyticus* isolates tend to be nonpathogenic, as indicated by the low detection frequency of common virulence factors including thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*), typically less than 10% prevalence of *tdh* or *trh*.[41] More than 80% of the *V. parahaemolyticus* isolates showed resistance toward ampicillin and cefazolin (Figure 5), which were also commonly observed. For example, previous studies in the Chesapeake Bay and Maryland Coastal Bays detected *V. parahaemolyticus* isolates showing a high percentage of resistance to penicillin (68%) and ampicillin (53%).[42]

5. CONCLUSION

V. parahaemolyticus is identified as the most prevalent *Vibrio* pathogen in the Ala Wai Canal based on amplicon sequencing and cultivation results. The unique physical configuration and hydrodynamic conditions of the canal are conducive for the enrichment of *V. parahaemolyticus* toward the dead-end terminus of the canal, due to high turbidity and nutrient levels in the stagnant water. The *V. parahaemolyticus* population in the Ala Wai Canal showed significant genetic diversity, suggesting the presence of diverse environmental niches. Fortunately, the *V. parahaemolyticus* population in the canal water appears to possess limited virulence factors and antibiotic resistance capabilities.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this article are available in GenBank database at https://www.ncbi.nlm.nih.gov/ and can be assessed with accession nos. ON965235−ON965243. Data are also available in the article and in its online Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.4c00368.

Additional experimental details, materials, methods, and results (PDF)

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Author Contributions

#D.Y.W.D. and P.S. contributed equally to this work and share first authorship. P.S. conducted sampling, performed lab experiments, and drafted the manuscript. D.D.Y.W. analyzed the data and revised the article. B.L. analyzed the Miseq sequencing data. M.K.J. performed statistical analysis and generated figures. T.Y. perceived the study and revised the article. All authors read and approved the final article. CRediT: **Doris Yoong Wen Di** formal analysis, writing-review & editing; **Min Ki Jeon** visualization, writing-review & editing.

Notes

The authors declare no competing financial interest.

https://doi.org/10.1021/acsestwater.4c00368
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■ ACKNOWLEDGMENTS

The authors would like to acknowledge Ms Misa Muranaka for helping with the water sampling and contributing to the *Vibrio* diversity analysis. This material is based upon work supported by the National Science Foundation under grant no. CBET-1855128.

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