Molecular Microbial Source Tracking of LBSP-Associated Fecal Indicating Bacteria in Saipan Coastal Waters for September 2017, March 2018, and August 2018.

MST Data Report of a Joint Study by the CNMI Bureau of Environmental & Coastal Quality, the NOAA Atlantic Oceanographic & Meteorological Laboratory, and American University.

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All samples identified as "BECQ" were collected by BECQ personnel as part of their water quality surveillance monitoring program during the time of this study. Samples identified as "KK" were collected by the team of Dr. Kiho Kim and Dr. Karen Knee from American University in Washington DC as part of an independent study on stable isotope source tracking in Saipan Lagoon during this same study period. We gratefully thank the crew from American University for their collection of additional samples of shore water, reef water, sediment, and ground water for us to use in our own microbial source tracking study while they were collecting samples for their own nitrogen isotope source tracking study.

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INTRODUCTION

The Commonwealth of the Northern Mariana Islands (CNMI) is a Commonwealth of the United States comprising all the islands of the Northern Mariana archipelago chain in the Northwest Pacific, except for the island of Guam. The capital and major population center of CNMI is the island of Saipan. Saipan, the largest of the Northern Mariana Islands, has a land area of 122 km^2 and is approximately 20 km long and 9 km wide. The island consists of a volcanic core enveloped by younger coral reef-derived limestone formations. The island has the most diverse types of coral reefs and associated habitats in the Commonwealth. A fringing and barrier reef system protects the majority of the beaches along the western and coastal plains. The western side of the island is the most populated and the coral reefs along these areas are negatively affected by human activity. Continuing sediment, nutrient, and microbial pollution, combined with stressors such as temperature-induced bleaching, affect many of Saipan's western and southeastern reefs.

Management of environmental and coastal quality in the Commonwealth, including water quality surveillance and management, comes under the jurisdiction of the CNMI Bureau of Environmental Coastal Quality (BECQ). BECQ is responsible for monitoring, assessing, and protecting water quality within the CNMI, as well as managing land, air, water, and coastal quality. Both Commonwealth and U.S. Federal laws and regulations mandate this responsibility (Yuknavage, 2018).

As a whole, CNMI's marine waters meet the high water quality standards designated by the CNMI BECQ. The majority of CNMI's marine waters are designated "Class AA" which reflects the highest water quality. However, there are still a variety of point and non-point sources of nutrient and microbial contamination that can lead to impaired waters and potentially affect both public health and ecosystem health, including coral reefs. For known point sources, only two sewage outfalls (Agingan and Sadog Tasi, Saipan) exist in the CNMI. Both sewage treatment plants are operated by the Commonwealth Utilities Corporation (CUC) and are designed to provide secondary treatment for an average daily flow of 3 and 4.8 million gallons per day, respectively. The Sadog Tasi's treated effluent is discharged through a marine outfall, approximately 365 m offshore into the Class A receiving waters off Tanapag Harbor, Saipan Lagoon at a depth of 15 m. The Agingan plant's treated effluent is discharged at the surf line through an intertidal outfall into the Class A receiving waters of Tinian Channel. In addition, Saipan has 18 underground injection wells used to dispose of reverse-osmosis (reject) brine. The injection wells belong primarily to tourist hotels located along the coast line of West Takpochau. The wells terminate below the freshwater/saltwater interface, and therefore do not pose a contamination risk to ground water withdrawn for consumption (Yuknavage, 2018).

There are also many non-point-source inputs of Land Based Sources of Pollution (LBSP) to the coastal waters of CNMI. In recent years, many microbiological violations of enterococci fecal indicating bacteria (FIB) levels occurred in areas with heavy stormwater runoff. Many of these sites were within the highly developed Garapan district, where drainage issues are in the process of being addressed. Other frequent violations occur within Saipan's marinas or in waters surrounding docks, or at high-density tourist swimming sites such as the "Grotto" (Yuknavage, 2018). Previous studies have shown that high densities of bathers at marine beaches can shed substantial levels of fecal indicator bacteria such as Enterococci and skin-associated bacteria such as Staphylococci (Elmir et al, 2009; Plano et al, 2011), as well as pathogens (Gerba, 2000).

In addition there are large numbers of dogs that roam the island, especially in developed areas around the central western lagoon shore, which can contribute significant loads of fecal bacteria to terrestrial runoff as non-point-source discharges. This dog fecal contamination may both represent a potential public health risk and may also confound water quality assessments based solely on general FIB measurements as dog feces can contain very high levels of enterococci (Solo-Gabriele, 2001; Cinquepalmi et al, 2013).

There are 240.5 miles of ocean shoreline in the CNMI, of which 50.5 coastline miles (21% of CNMI coastline miles) were found to be impaired due to exceedance violations of Enterococci FIB levels in the year 2018, as reported by BECQ in the 2018 CNMI 305(B) and 303(D) Water Quality Assessment Integrated Report (Yuknavaage, 2018). Of these, 32.7 miles of enterococci-impaired coastline surround Saipan (**Figure 1**). As in previous years, the most common sources of Enterococci contamination were from point sources, such as failing sewer lines and other municipal wastewater collection, or individual on-site wastewater collection systems, and non-point sources (NPS). NPSs include: 1) sediment-laden storm water runoff with naturally occurring Enterococci from urban runoff, secondary coral roads, erosion from construction sites and new developments, etc.; 2) Illicit wastewater discharges from animal pens and outhouses; 3) waste from free-range feral and domestic livestock; and 4) in the case of remote tourist locations, an increase in visitor numbers in conjunction with a lack of available public restroom facilities at these sites. In **Figure 1**, note particularly the prevalence of impaired water listings involving Enterococci FIBs at beach sites along the coastline of the western Saipan Lagoon region (Yuknavage, 2018).

The BECQ regularly monitor the microbiological water quality of coastal CNMI waters during their routine Water Quality surveillance program, testing for Enterococci fecal bacteria with the IDEXX EnteroLert testing system (IDEXX Laboratories) and for *Escherichia coli* with the IDEXX ColiLert testing system (IDEXX Laboratories) as per the manufacturer instructions and EPA guidelines. Enterococci and *E. coli* enumeration by live culture methods using either the mEI agar plate test method (EPA method 1600) or the commercial IDEXX Most Probable Number Chromogenic Substrate Method (EnteroLert and ColiLert) are the only regulatory promulgated bacterial water quality criteria and methods for environmental recreational waters in the US. However, the US EPA has provided an alternative molecular method based on quantitative PCR (qPCR) of ribosomal 16S genes specific in Enterococci spp. (EPA method 1611) that US States and Territories may adapt for use in developing alternative criteria and/or supplemental testing. However, these current Fecal Indicating Bacteria are general FIBS that can be found in many animal species or other environmental sources and are not exclusive to the human gut, thus they are not exclusive indicators of human-source feces that is considered the high risk for fecal contamination of environmental waters. The qPCR testing of enterococci for water quality assessment, particularly based on the Entero1A (Haugland et al, 2005; Haugland et al, 2016) assay, which is now incorporated into the EPA method 1611 (US EPA, 2012), is gaining more wide spread use, resulting in various commercially-available kits and instrumentation for conducting such qPCR assessments. However, both the traditional culture based and the qPCR based tests for general Enterococci do not have a source-tracking capability and do not discriminate the animal host fecal sources the detected enterococci FIBs may have come from. Thus, such enterococci monitoring can be useful for identifying “hot spots” and zones of water quality exceedance, but may be of more limited utility in helping resource managers understand why there are such exceedances or where the microbial contamination may be coming from.

Figure 1: Saipan Coastal Consolidated Assessment and Listing Methodology (CALM) Categories and the causes of impairment. Note especially the coastline areas considered impaired for Enterococci exceedance. Source: Yuknavage et al. (2018), "2018 CNMI 305(B) and 303(D) Water Quality Assessment Integrated Report" by CNMI BECQ.

Many strains and species of gut microbial flora have co-evolved with their animal hosts and contain unique gene sequences that can be diagnostic for particular FIBs indicating fecal contamination specific to particular host animals (such as humans, dogs, birds, pigs, cows, etc.). Therefore, being able to enumerate the relative abundance of such host-specific fecal indicating bacteria can be a useful tool for resource managers to help investigate "hot spots" of microbial contamination and potentially provide better insight into the possible sources and patterns of transport for LBSP that might be contributing to the microbial contamination affecting impaired waters. Patterns of excessive human-source fecal indicators can suggest infrastructure problems, sanitary leakage, etc., while targets such as canine-source FIB markers may indicate increase contamination inputs from surface runoff sources, and of course various agricultural FIB markers can draw attention to closer inspection of possible livestock fecal inputs, while excessive bird marker may indicate background inputs from wildlife populations. Such Microbial Source Tracking (MST) can add another set of tools to the resource management toolbox which, in combination with other methods, may help better direct investigation and mitigation efforts. Microbial Source Tracking, or MST, is a DNA-based technology that enables the water-quality management community to determine whether humans or other animal species are responsible for microbial fecal contamination in an aquatic environment. Beach water-quality managers use microbial source tracking – also known as microbial source identification – to gain insights into the degree of health risk posed by fecal contamination at a given site; human fecal matter is far more likely to be infectious to humans than the feces of seagulls, livestock and most other animals.

A variety of methods for molecular Microbial Source Tracking of fecal indicator bacteria have been developed, tested, and deployed, and applications for MST in water quality management are becoming increasingly common. Note that MST methods are not yet promulgated for regulatory criteria and there are currently no abundance threshold exposure limits promulgated for regulatory purposes for any host-specific MST genetic markers. Rather, MST assessment of the relative abundance of host-specific fecal bacterial genetic markers are currently utilized more commonly in conjunction with the regulatory general FIB assessments for enterococci as a troubleshooting approach to investigate chronic microbial water quality problems. This type of study can be highly effective when integrated into a multi-tool, multi-tiered approach for water quality assessment, as described in the "The California Microbial Source Identification Manual: A Tiered Approach to Identifying Fecal Pollution Sources to Beaches" (Griffith et al, 2013) by the Southern California Coastal Water Research Project (SCCWRP). This manual describes how a multi-tiered integrated microbial source tracking study can be organized and also provides specific molecular protocols that have been validated by multi-lab performance testing for conducting qPCR MST assays to detect a variety of host-source specific fecal indicating bacteria genetic markers.

The Molecular and Environmental Microbiology Program at the NOAA Atlantic Oceanographic and Meteorological Laboratory (AOML) is part of the national laboratory system of the National Oceanic and Atmospheric Administration (NOAA) Office of Oceanic and Atmospheric Research (OAR). NOAA AOML is one of the recognized leading national labs that is developing, deploying, and conducting technology transfer training of qPCR-based MST methods. AOML works closely with partner labs in federal agencies (especially the EPA), State /Territorial agencies, local county/city agencies, and in academia to develop, test, and teach others such MST technology. AOML has been a participant lab in the multi-laboratory validation testing of MST assays with both the US EPA and with SCCWRP. AOML conducts a

robust Technology Transition program in MST methods, and has helped to set up and train molecular microbial source tracking facilities at other agencies, including the Florida Department of Environmental Protection, the Broward County Environmental Monitoring Laboratory, and now with the CNMI Bureau of Environmental and Coastal Quality.

In September of 2017, in March of 2018, and again in July/August of 2018, NOAA AOML personnel traveled to Saipan to modify and adapt the Pall GeneDisc qPCR system that was already at BECQ to be able to run MST assays, and to train BECQ lab personnel on select MST methods from the SCCWRP California Source Identification Manual for human-source *Bacteroides* (HF183 assay and the EPA Hum-M2 assay), dog-source *Bacteroides* (DogBact assay), cow-source *Bacteroides* (Cow-M2 assay), pig-source *Bacteroides* (Pig2Bact assay), and seagull/seabird-source *Catellicoccus marimammalium* (Gull2 assay). This MST Technology Transition to CNMI BECQ was part of the NOAA Coral Reef Conservation Program, CRCP Project 31184. The transitioned MST technology was then used by BECQ to conduct an MST study of host-specific fecal indicating bacteria associated with LBSP in Saipan coastal waters during the wet and dry season of 2018, with personnel from both NOAA AOML and American University in Washington DC participating in the study. Travel and sample analysis for the LBSP MST study in Saipan was funded by both NOAA project CRCP 31184 and by the NOAA AOML 'Omics Initiative.

MST Study of LBSP Host-Specific Fecal Indicators in Saipan Coastal Waters

Field Program Overview and Sampling

The field program for this study was conducted from March 12-19 2018 and from July 31 to August 8 2018. There were also a few preliminary samples collected on September 13 2017, but these were primarily samples for demonstration and were used to teach MST methods to BECQ personnel during the first CRCP project 31184 Technology Transition Workshop for MST in Saipan in September of 2017. All of the samples collected, along with results of the MST analysis are shown in the final MST data table in **Appendix 1**. Locations of the sample sites are show in **Figures 2A-2C,** with the GPS coordinates of the sample sites listed in **Table 1A** and **Table 1B**. The field program sampling consisted of two parts: (1) regular BECQ water quality surveillance samples collected as part of the on-going water quality monitoring program – these samples include the term "BECQ" in the sample name; and (2) special sampling conducted by team of Dr. Kiho Kim from American University for nitrogen isotope source tracking and radon measurements – the additional samples they collected for us to analyze by MST include the term "KK" in the sample name. On the map in **Figure 2**, site numbers starting with "NEB" (for north east beach), "SEB" (for south east beach), or "WB" (for west beach) are part of the regular BECQ water surveillance program sites and correspond to their regular BECQ site names. Sites with numbers starting with "S" (for shore), "R" (for reef), or "LL" (for Lao Lao Bay) were collected by American University as part of the "KK" samples. Ground water and sediment samples correspond to the same location as the same numbered shore site (for example, S18,

GW-S18, and sedGW-S18 are shore water, ground water, and beach sediment samples respectively taken at the same S18 shore site).

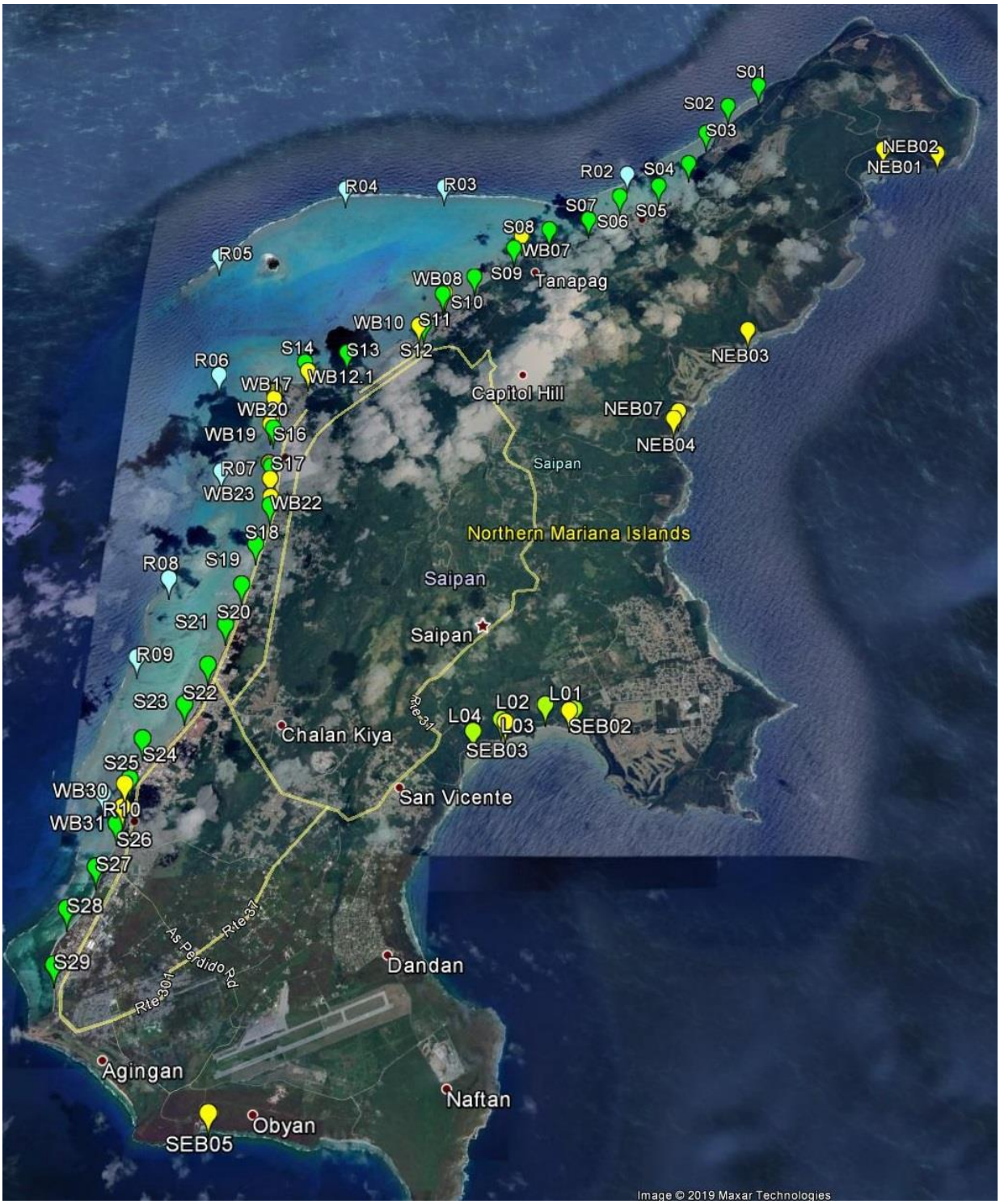


Figure 2A: Location of all sample sites used for MST analyses. "NEB", "SEB" & "WB" were collected by BECQ, "S", "R", & "LL" were collected by American University.

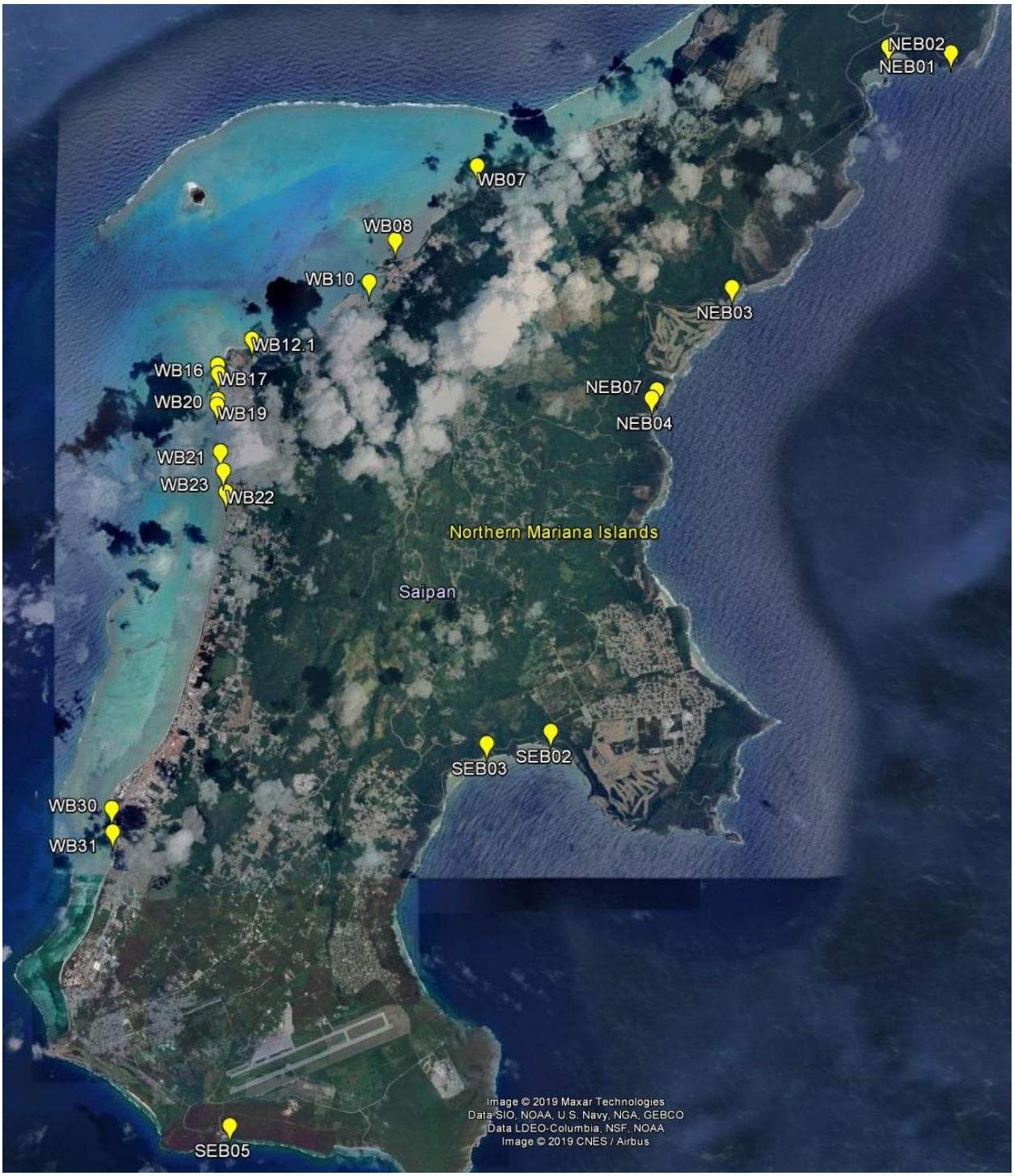


FIGURE 2B: Location of sample sites collected by BECQ used for MST analyses.

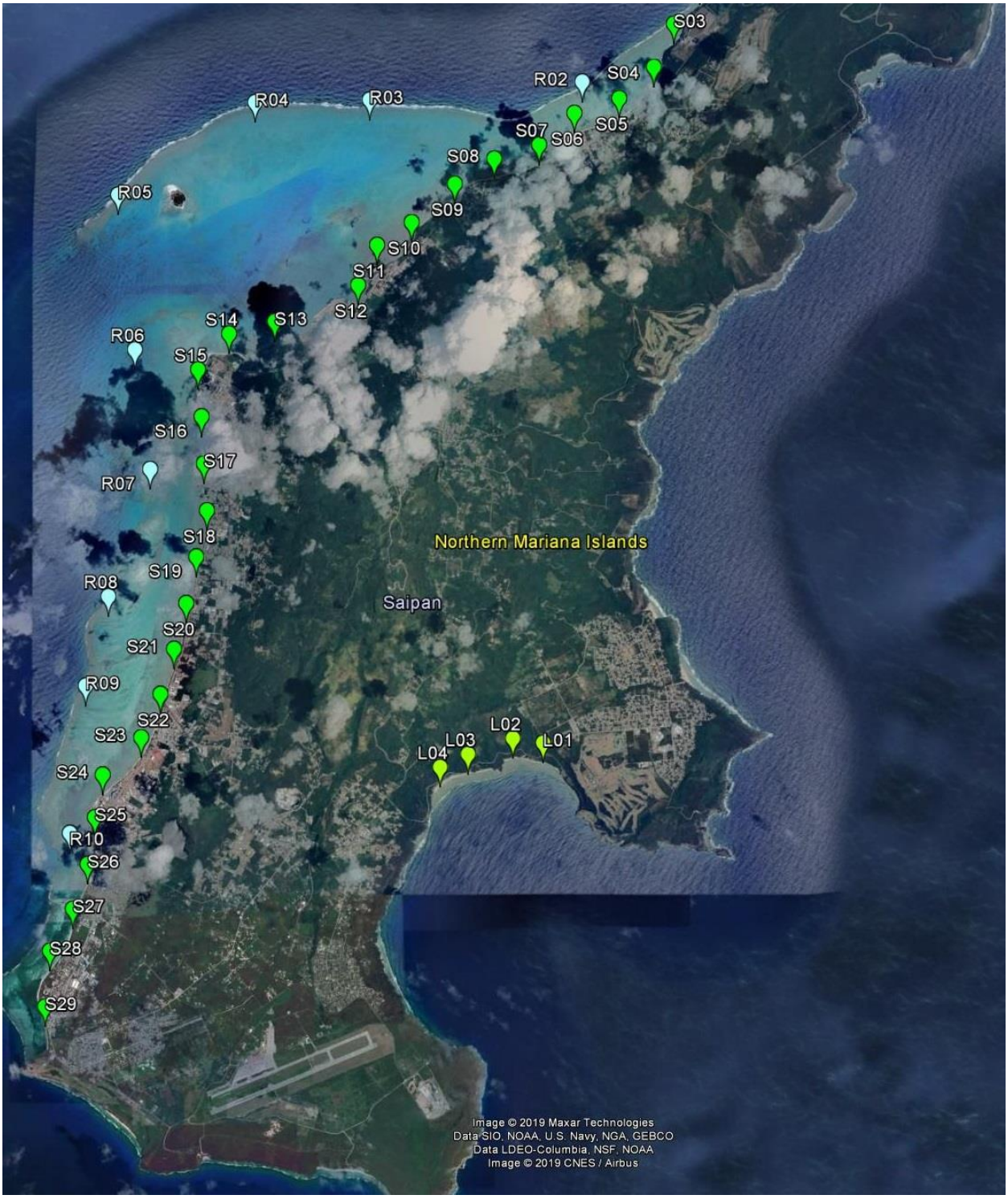


FIGURE 2C: Location of sample sites collected by American University used for MST analyses.

Table 1A: GPS Coordinates for Saipan MST study sample sites collected by American University Personnel during their Nitrogen Isotope source tracking study. Site labels correspond to the AU study site IDs.

Sample Site ID	BECQ site name	Latitude	Longitude
American University Sample Sites in Saipan used for MST			
KK-S01	x	15.272307	145.792983
KK-S02	x	15.268146	145.787746
KK-S03	x	15.262718	145.78402
KK-S04	x	15.256746	145.781119
KK-S05	x	15.252371	145.776126
KK-S06	x	15.250323	145.769663
KK-S07	x	15.245921	145.764582
KK-S08	x	15.243969	145.758113
KK-S09	x	15.240486	145.75242
KK-S10	x	15.235147	145.746218
KK-S11	x	15.231979	145.741222
KK-S12	x	15.22641	145.738477
KK-S13	x	15.221355	145.726457
KK-S14	x	15.219648	145.719871
KK-S15	x	15.214674	145.715417
KK-S16	x	15.208152	145.715918
KK-S17	x	15.201604	145.716251
KK-S18	x	15.195086	145.71668
KK-S19	x	15.18867	145.715166
KK-S20	x	15.182211	145.713697
KK-S21	x	15.175859	145.711938
KK-S22	x	15.169579	145.70999
KK-S23	x	15.163536	145.707222
KK-S24	x	15.158366	145.701672
KK-S25	x	15.152443	145.70056
KK-S26	x	15.145947	145.69951
KK-S27	x	15.139724	145.697355
KK-S28	x	15.133885	145.694066
KK-S29	x	15.126143	145.693372
KK-R02	x	15.254677	145.770817
KK-R03	x	15.252171	145.740146
KK-R04	x	15.251834	145.723624
KK-R05	x	15.238982	145.703865
KK-R06	x	15.217466	145.706273
KK-R07	x	15.200849	145.708484
KK-R08	x	15.183176	145.702492
KK-R09	x	15.170759	145.699209
KK-R10	x	15.150247	145.696933
KK-L01	x	15.162799	145.765156
KK-L02	x	15.163437	145.760804
KK-L03	x	15.161266	145.754351
KK-L04	x	15.159449	145.75032

Table 1B: GPS Coordinates for Saipan MST study sample sites collected by BECQ personnel from their regular CNMI BECQ Water Quality Surveillance Program sample sites. Site labels correspond to the regular BECQ WQS site IDs.

Sample Site ID	BECQ site name	Latitude	Longitude
BECQ Water Quality Surveillance Sample Sites in Saipan used for MST			
BECQ-NEB01	Grotto	15.25872359	145.82319891
BECQ-NEB02	Bird Island	15.25956572	145.81402146
BECQ-NEB03	Jeffrey's Beach	15.22544400	145.79102700
BECQ-NEB04	Old Man by the Sea	15.20973484	145.77922334
BECQ-NEB07	Hidden Beach	15.13320000	145.47240000
BECQ-WB07	Tanapag Meeting Hall	15.24269986	145.75359491
BECQ-WB08	Central Repair Shop	15.23218678	145.74155397
BECQ-WB10	DPW Channel Bridge	15.22625876	145.73769149
BECQ-WB12.1	American Memorial Park Drain	15.21811662	145.72048989
BECQ-WB16	Dai Ichi Hotel	15.21447578	145.71547442
BECQ-WB17	Drainage #1 (Dai Ichi)	15.21323831	145.71556180
BECQ-WB19	Hafa-Adai Hotel	15.20955485	145.71544512
BECQ-WB20	Drainage #2 (Hafa-Adai Hotel)	15.20882265	145.71538005
BECQ-WB21	Garapan Fishing Dock	15.20218380	145.71586838
BECQ-WB22	Garapan Beach	15.19647489	145.71667415
BECQ-WB23	Drainage #3 (Garapan)	15.19946847	145.71630856
BECQ-WB30	Sugar Dock	15.15161651	145.69991513
BECQ-WB31	CK Dist #2 Drain	15.14827499	145.70005013
BECQ-SEB02	North Lao Lao Beach	15.16256974	145.76436653
BECQ-SEB03	South Lao Lao Beach	15.16084846	145.75497900
BECQ-SEB05	Ladder Beach	15.10665252	145.71725701

METHODS

Sample Collection, Filtration, and Preservation:

Water samples intended specifically for molecular MST analysis were collected in sterile 1.5 L Whirlpak bags (Nasco), then held on ice and transported back to the BECQ laboratory and filtered within 6 hours of initial sample collection. At the BECQ laboratory in Saipan, 1 L aliquots of sample were filtered through sterile 0.45 micron, 47mm diameter cellulose ester filters (Pall GN6, Pall Corporation) using disposable sterile filter funnels (Pall Microfunnel, Pall Corporation). In some cases a smaller volume was filtered for samples where the water was too turbid to allow filtration of 1 L. In all cases, the actual volume filtered was recorded and used in subsequent calculations for MST target copy number. For the training demonstration samples used in September of 2017 and for the groundwater samples used in August of 2019, water sample were filtered through the sterile 0.4 micron, 47mm diameter polycarbonate filters that come as part of the Pall disposable Microfunnels (Pall Corporation). Sample filters were rolled

used flame-sterilized forceps and aseptically transferred to “Lysing Matrix E” bead beat tubes (from the FastDNA Spin Kit for Soil, MPBiomedicals). Filters in these tubes were then preserved by adding approximately 2 mL of the DNAgard-Tissue preservative solution and stored frozen until later DNA extraction and purification. The exception to this were the original training demonstration sample filters from September 2017, where the polycarbonate filters were placed in empty sterile 2mL tubes and stored frozen until extraction, without the use of any addition DNA preservative solution.

FIGURE 3: Dean Palacois of BECQ (foreground) and Dr. Maribeth Gidley of UM-CIMAS (Background) in the BECQ lab at Saipan preparing environmental DNA extracts for MST analyses in March 2018

Most of the DNA sample filters were extracted and purified at the BECQ laboratory in Saipan, however the samples collected on March 19, 2018 and on August 7-8, 2019 were preserved in DNAgard and transported back to the NOAA-AOML laboratory in Miami, where they were extracted and purified. The same DNA purification method was use both in the Saipan BECQ lab and in the Miami NOAA-AOML lab for all samples from the 2018 sampling events. Note that the samples taken to Miami spent approximately 3 days at room temperature during transport in carry-on baggage via commercial airline, but the DNAgard-Tissue preservative solution (Biomatrica) stabilizes the DNA during room-temperature storage, and this time-frame is well within the effective room-temperature storage-life for DNAgard-preserved samples (Biomatrica). Upon arrival at the Miami NOAA-AOML lab, the sample filters were then frozen until subsequent DNA extraction and purification.

In the case of the 5 beach sediment samples collected during the American University groundwater sampling during the last day of the project, 1 gram samples of sand were placed

into 2mL sterile screw-cap centrifuge tubes and preserved by the addition of approximately 1.5mL of DNAgard-Tissue DNA preservative solution (Biomatrica). These preserved sediment tubes were then transported back to the NOAA-AOML laboratory and stored frozen at -80°C, where they currently remain stored. As of the time of this writing, these 5 sediment samples have not yet been extracted, purified, or analyzed and remain archived in cryo-storage pending later extraction and analyses.

Live Enterococci FIB Enumeration via the Commercial IDEXX EnteroLert Method:

Live Enterococci were measured using the standard EPA-approved regulatory method of the IDEXX EnteroLert™ Chromogenic Substrate method, following EPA guidelines, established BECQ protocols, and manufacturer instructions. All samples analyzed by IDEXX EnteroLert were done at the BECQ laboratory in Saipan. All samples collected by BECQ personnel and most of the samples collected by American University personnel were done by BECQ personnel using BECQ supplies and BECQ IDEXX equipment. IDEXX EnteroLert analysis of some samples collected by American University personnel were done by American University personnel while at the BECQ laboratory using their own EnteroLert consumable supplies but with the BECQ IDEXX equipment. Not all of the data set from the IDEXX EnteroLert analysis has been communicated with AOML as of the time of this writing, so a more complete set of EnteroLert data may be available by contacting the BECQ laboratory directly. The viable Enterococci abundance in MPN/100mL as estimated by IDEXX EnteroLert test is shown in the study data table of **Appendix 1**. The label "?" indicates a sample for which EnteroLert was either not done or the EnteroLert data for that sample was not provided to AOML as of the time of this writing.

Extraction and Purification of Total Microbial Community Genomic DNA from Samples:

For the twelve MST Workshop training demonstration samples that were collected in September of 2017 on 0.4 um polycarbonate filters, extraction and purification of total genomic DNA was conducted by the protocol of the GeneDisc Ultra-Purifier Extraction System (Pall Corporation) as per manufacturer instructions for GeneDisc Recreation Water E. coli and Enterococcus spp. assay kit (Pall, Corporation). This was conducted on the Pall Extractor System equipment in the BECQ lab (shown in the background of **Figure 4**) using the Extraction Pack Environment 1 Kit (Pall Corporation). In brief, the sample filter was aseptically transferred to a lysis tube from the extraction kit, using flame-sterilized forceps, then sonicated in kit lysis buffer, heated at 110°C for 20min. The lysate was then filtered under vacuum onto a silica DNA-binding column from the kit in the Pall Ultra-Purifier instrument to bind the DNA and then washed twice with the kit washing buffers #1 and #2 under vacuum. The column was dried and then the bound purified DNA was eluted from the silica binding column with a total of 200uL of

pre-heated elution buffer from the kit. The eluted DNA was collected under vacuum into the final DNA recovery tubes from the kit, and the purified genomic environmental DNA extract was stored frozen at -20°C until analysis.

Figure 4: Dean Palcois of BECQ setting up molecular MST reactions. The Pall GeneDisc DNA Extractor system at the BECQ lab can be seen in the background.

For all of the regular Saipan LBSP MST Study samples from March and September 2018 collected and stored on 0.45 um cellulose ester filters, the extraction and purification of total genomic DNA was conducted by the protocol of the FastDNA Spin Kit for Soil (MPBiomedicals, Thermo-Fisher) according to manufacturer instructions with minor modifications as follows. Filters were stored until processing in "Lysing Matrix E" bead tubes from the kit (MPBiomedicals) filled with DNAgard-Tissue preservative solution (Biomatrica) as described above. In the case of frozen filters, the samples preserved in the bead-beat tubes were completely thawed first. Tubes were centrifuged down for 5 minutes as 12,000xg, and the majority of the DNAgard preservative solution was gently pipetted off and discarded without disturbing the filter, beads, or cell pellet. The rest of the extraction processing was as per the kit instructions. In brief, 978uL of the kit sodium phosphate buffer and 122uL of the kit MT lysis buffer were added to the bead-beat tubes with the filter, and the cell samples on the filter were then lysed and homogenized by vigorous bead-beating using the hand-held beat-beating homogenizer instrument "Super FastPrep-2" (MPBiomedicals) as per manufacturer instructions at a speed setting of 20 for a duration of 5 seconds. This gives equivalent bead-beating homogenization as to that achieved using a standard benchtop bead-beating instrument such as a "FastPrep-24" (MPBiomedicals) using an impact speed setting of 6 m/s for 60 seconds (however the hand-held Super FastPrep-2 can only process two tubes at a time). After that, the DNA from the supernatant is bound to the kit's Binding Matrix column, washed twice with the kit's wash buffer, and then finally eluted with heated elution buffer from the kit. Binding columns were eluted with a total of 80uL of elution buffer into kit recovery tubes, then the eluted DNA was stabilized for room temperature storage by the addition of 20uL of DNA-Stable-Plus preservative solution (Biomatrica), giving a final extracted elution volume of 100uL. These elutions were subdivided into replicate aliquots, so that part of the DNA samples remained stored at the BECQ lab in Saipan and replicate aliquots of the DNA samples were transported back to the NOAA-AOML lab in Miami. Samples were kept stored frozen until analysis, except for the brief period of time of a few days when samples were being transported at room temperature from Saipan

back to the NOAA-AOML lab in Miami (with the room-temperature protection of the DNA-Stable-Plus) preservative.

Modification of MST qPCR assays to run on the Pall GeneDisc qPCR Thermocycler Instrument:

To date the established molecular MST protocols as described in the SCCWRP Microbial Source Identification Manual (**Griffith et al, 2013**), have been deployed using typical research-grade quantitative real-time PCR thermocylers that are highly customizable, and a variety of such platforms have been tested and validated in a number of multi-lab trials. Current MST methodology and protocols require substantial laboratory facilities and resources and personnel who are highly trained in the methodology and protocols, and utilizes extensive pipetting and reagent preparation, and molecular reaction setup. It is not a simple nor user-friendly process, and requires high precision in the setup and handling of the reagents and reference standards needed to properly conduct the assays, along with a variety of both positive and negative controls, and appropriate scientific interpretation of both the sample and control results.

Figure 5: The Pall GeneDisc Rapid Microbiology System Thermocycler

Conversely, the Pall GeneDisc qPCR Rapid Microbiology System thermocycler (**Figure 5**, Pall Corporation) is specifically designed to be very simple and user-friendly but by its nature this cycler is not typically customizable. This is a commercial applications thermocycler rather than the more versatile but complicated research-grade thermocyclers, and the GeneDisc thermocycler is an automated system designed to run pre-prepared GeneDisc plate kits with all reagents, primers, probes, and other assay components already loaded and preserved in the plate wells, so the end-user only has to add their DNA samples to the plate and scan the barcodes, and the GeneDisc system does everything else in an automated fashion, controlling the qPCR reactions, measuring the target abundances, and calculating and interpreting the results automatically for the end-user. This system has primarily been developed for quality control in the commercial food and beverage industry, and requires minimal laboratory facilities and minimal personnel training. While most of the developed commercial assays for this system are for typical food/beverage contaminants and pathogens, the company does also have a commercial recreational water quality plate available that measures Enterococci and *E. coli*, based on the standard general qPCR assays available in the public domain that have been developed by the US EPA (i.e. the same enterococci 16S ribosomal gene target utilized in EPA Method 1611). However, due to the nature of the instrument, the end-user is typically prevented from running any customized assay or thermocycling protocols, and the GeneDisc system at the time of this BECQ MST study in

Saipan did not have any commercially available MST assays that could run on a GeneDisc format.

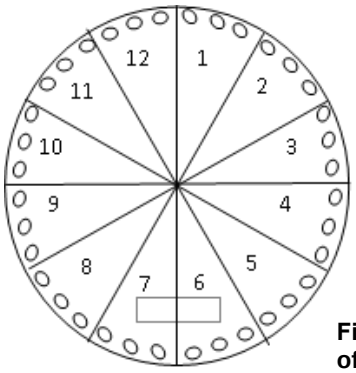
The CNMI BECQ environmental quality laboratory in Saipan had acquired one of these Pall GeneDisc systems (along with an associated Pall GeneDisc DNA Extractor system) about a year prior to the start of the LBSP MST study reported here. The BECQ laboratory had used this Pall GeneDisc system along with the commercial PALL GeneDisc recreational water quality assay kit to test for both Enterococci and E.coli as proxy markers for contamination in Saipan coastal waters. However, as described above, their GeneDisc system had no capability of conducting actual source tracking for samples found to contain excess fecal indicators, or for identifying host-specific fecal contaminants, due to the constraints of their commercial qPCR thermocycler system and their lack of training in state-of-the-art MST methodologies.

The primary goal of the NOAA Coral Reef Conservation Program Project # 31184 (with Principal Investigator Christopher Sinigalliano) was to conduct a technology transition of this MST technology to the CNMI BECQ laboratory in Saipan by helping them to adapt their available Pall GeneDisc instrument to be able to conduct custom MST qPCR thermocycling and to train members of their BECQ laboratory personnel in how to properly perform such molecular microbial source tracking analysis with their own equipment in-house at the BECQ environmental laboratory facility in Saipan. The goal of the subsequent NOAA-AOML 'Omics Initiative project was to help support the BECQ in the implementation of this new MST analytical capability to conduct an MST study of LBSP patterns of host-specific fecal indicating bacterial in Saipan coastal waters, especially Saipan Lagoon, to help aid local BECQ management efforts in coral reef protection and management. The results of that MST LBSP study are reported here. NOAA-AOML formed a collaborative research partnership with CNMI BECQ and with the Pall Corporation GeneDisc Division for research and development to adapt the GeneDisc instrument at the BECQ laboratory for conducting such MST assays. Pall GeneDisc developed and made available special "open" or "blank" GeneDisc plates that did not have pre-prepared reagents load in them, and modified the instrument software to allow AOML and BECQ personnel to bypass the typical commercial end-user restrictions and be able to custom program the cycler to run the specific conditions necessary to perform the MST assays as per the SCCWRP California Microbial Source Identification Manual protocols (as appropriately modified by AOML to run on the format and size of the GeneDisc plate wells). A new set of BECQ MST protocols was established (based on the SCCWRP protocols) specifically for running the following MST assays on the BECQ GeneDisc instrument in Saipan: (1) Human-source Bacteroides assay "HF183"; (2) Human-source Bacteroides assay "HumM2"; (3) Dog-source Bacteroides assay "DogBact"; (4) Cow-source Bacteroides assay "CowM2"; (5) Pig-source Bacteroides assay "Pig2Bact"; and (6) the "Gull2" assay specific for *Catellicoccus marimammalium* fecal bacteria found in the gut of most seagulls, as well as potentially other birds (especially seabirds) that may co-habit, scavenge, or nest with seagulls (depending on geographic location this may include species of terns, pelicans, geese, and very often pigeons).

qPCR MST Reaction Setup on GeneDiscs:

Quantitative PCR reactions for MST assays of human, dog, pig, and cow Bacteroides and of seagull/seabird Catellicoccus were set up as per the SCCWRP California Microbial Source Identification manual (Griffith et al, 2013) , with the following modifications. Total reaction volumes were 12uL per plate well, set up as sectors for 3 plates, where 30 uL of working reaction cocktail were mixed with 6 uL of target sample DNA and pipetted into the respective sample sector of a "Blank" GeneDisc plate type 01MT, which then filled 3 replicate plate wells under vacuum with 12 uL of the sample/reaction cocktail mixture (**Figure 6**). All assays had been modified and optimized to work in the final PCR reaction at a final forward and reverse primer concentration of 1uM and a final probe concentration of 80nm in 1X of the Pall Corporation GeneDisc Mastermix (Pall Cat# SR008), and reactions sealed in the well with sterile mineral oil as per the manufacturer instructions. The thermocycler was then run with the proprietary Pall Genefile plate file 01MT_0B that is specific for the custom run of the "blank" MST GeneDiscs type 01MT. This represents cycling conditions of 15 minutes at 95°C to activate the hotstart polymerase enzyme of the mastermix, followed by 40 repetitive cycles of 95°C denaturation step for 15 sec followed by 60°C annealing and extension step for 1 min, with a fluorescence reading of FAM and ROX dye in each well at the end of each extension step. The instrument recorded the Cq cycle values from the raw fluorescence values, but since the instrument software was not designed to automatically generate standard curves and do quantity calculations for the non-commercial "Blank" MST assays with the on-board instrument software, the fluorescent read data and Cq Cycle Threshold data were exported as .csv files from the instrument and imported into spreadsheet format in Microsoft Excel. Standard curves of the Cq values versus the Log10 value of the standard DNA concentrations were then plotted with the R statistical package and the final mean quantities from the 3 replicate wells for each environmental samples or control sample were then calculated in target sequence copies per reaction based upon the Cq, and the slope and intercept of the linear regression of the target DNA positive control concentration standards.

$$\textit{Mean log10 copies per reaction} = \frac{(\textit{sample mean Cq} - \textit{intercept})}{\textit{slope}}$$



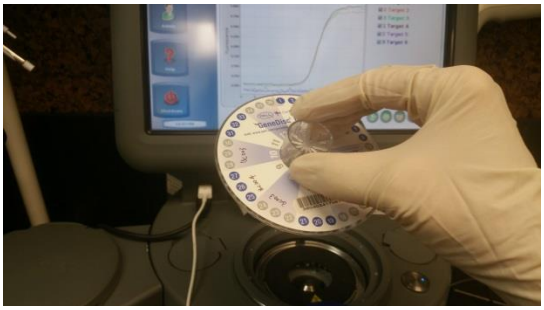


Figure 6: Loading a "blank" MST GeneDisc. The GeneDisc Plate consists of 12 sectors containing 3 wells per sector. By depositingng the PCR mix and the DNA sample in a sector (36µL final volume per sector), sample is analyzed in triplicate (12µL final volume per well).

RESULTS

The detailed results of all Microbial Source Tracking assessments for this study are shown in the final project data table in **Appendix 1,** and summarized in **Figures 7-10**. The results of the associated Nitrogen Istope source tracking and groundwater Radon study by the American University research group have been reported elsewhere (see Kim et al, 2019, Final Report to NOAA CRCP). Our MST study with the CNMI BECQ in Saipan found that both human and dog fecal bacterial genetic markers were relatively widespread, while seagull/seabird marker appeared to be somewhat more localized. Both agricultural markers for cow and pig fecal bacteria were much lower than had been anticipated and were only rarely seen during this study, and never above background DNQ (detected by not quantified) levels. The reason for the lower than expected detection of these agricultural markers is not clear, and further detection sensitivity testing of the cow and pig markers for local Saipan livestock populations may be warranted. For cows in particular, the US EPA has determined that detection of the cow-specific Bacteroidales marker "CowM2" may vary somewhat by region depending on the diet and age of the cattle (young calves may not have developed the target populations in the ruman yet).

59 samples out of 182 total samples (32.4%) demonstrated levels of human-source fecal Bacteroides marker at over 100 target sequence copies per 100mL. Of these, 11 samples out of 182 (6.0%) were over 1000 tsc/100mL for the human fecal Bacteroides marker (Figure 7). While there are no regulatory thresholds or exposure guidelines for these human FIB markers, we suggest here that such levels over 1000 target sequence copies per 100mL might reflect potential public health risk from exposure. In addition, a recent study on Quantitative Microbial Risk Assessment (QMRA) was conducted for human fecal contamination of various ages in environmental waters as measured by the human fecal Bacteroides marker HF183 (Boehm et al., 2018). This study relates that swimmer exposure to sewage after it has aged ~3 days results in median risks less than 30/1000 illnesses. They suggest a water quality threshold of the HF183 human Bacteroides marker of 1000 copies per 100 mL for exposure to a

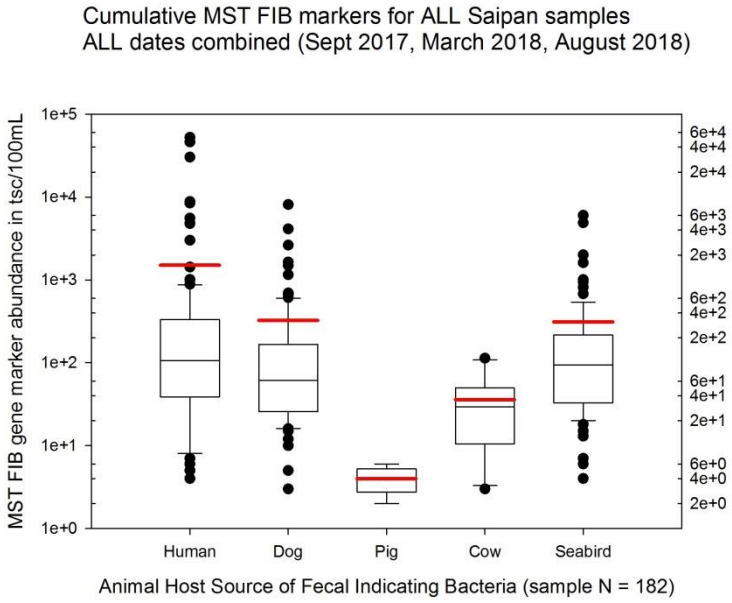


Figure 7: Boxplots summarizing the overall results of MST analysis for host-source fecal indicator bacteria 16S ribosomal gene marker abundance in Saipan coastal water for all water samples collected during the study for MST from September 2017 through August 2018. Black horizontal bars indicate the median, and red horizontal bars indicate the mean.

fresh known sewage source of an age of 3 days or less. However, this study recommends that exposure to sewage contamination that is greater than 3 days age or of uncertain age should utilize a risk-based water quality threshold for the HF183 human Bacteroides gene marker in surface waters that takes into account uncertainty in contamination age – the authors' QMRA-derived exposure threshod for human fecal contamination of uncertain age was determined to be 4100 copies per 100 mL of HF183 marker in surface water.

In our study of Saipan waters, 3 samples showed human FIB marker at over 10,000 copies per 100mL, which may reflect a serious exposure risk to public health and might be suggestive of significant leakage of sanitary infrastructure at those sites on those sample dates. It should be noted that all of these extreme exceedances of the HF183 marker at over 10,000 tsc/100mL were all relatively close to each other (the American University KK sites S20 and S18), they occurred within a few days of each other in the region of the western central Saipan Lagoon shoreline. Two of these extreme exceedance samples were actually groundwater samples from the same site S18 collected on August 3rd and August 7th respectively of 2018. The third extreme level of human FIB marker was observed for the shoreline sample of American University KK site S20 collected on August 2nd 2018 (**Figure 8, Figure 9**).

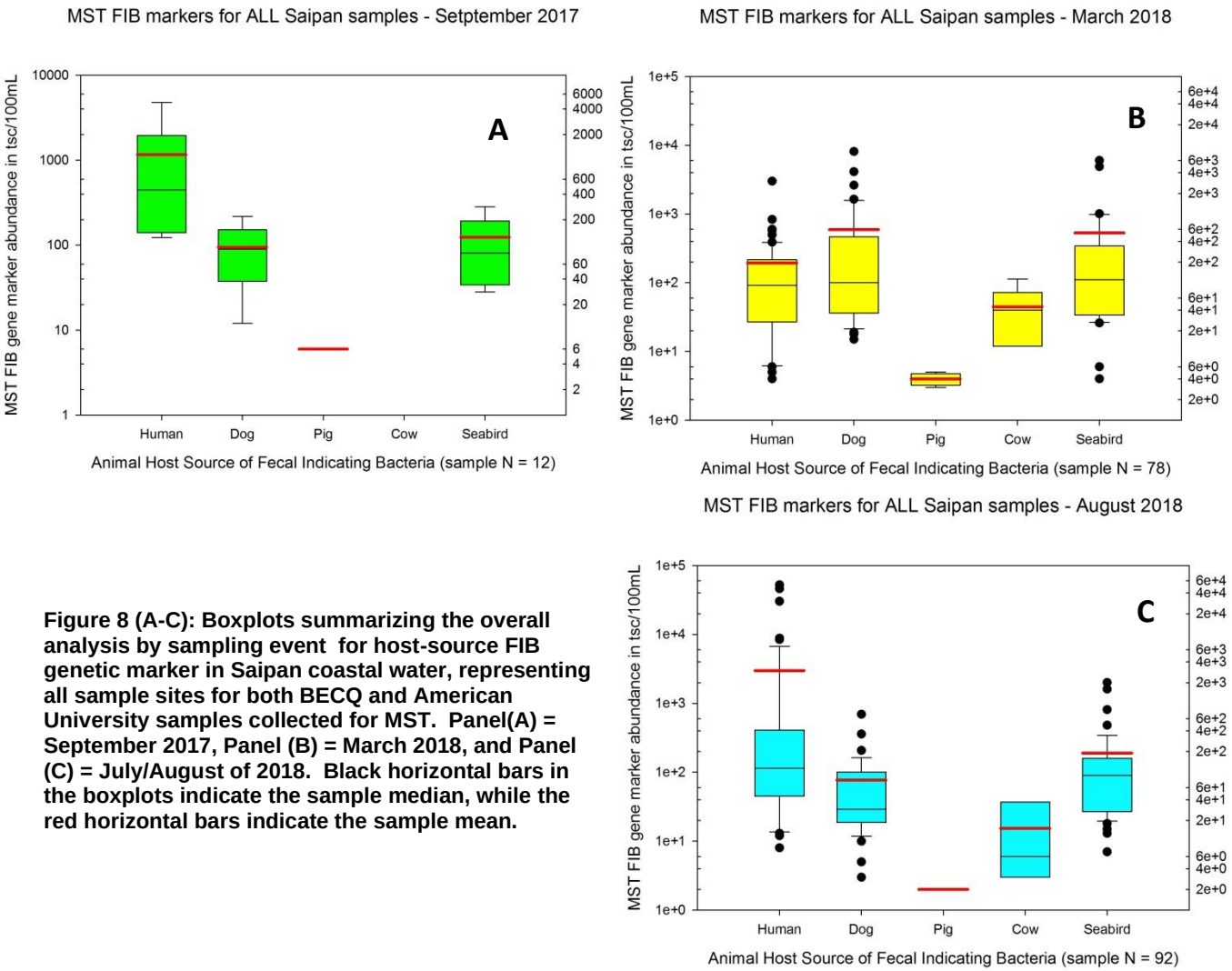


Figure 8 (A-C): Boxplots summarizing the overall analysis by sampling event for host-source FIB genetic marker in Saipan coastal water, representing all sample sites for both BECQ and American University samples collected for MST. Panel(A) = September 2017, Panel (B) = March 2018, and Panel (C) = July/August of 2018. Black horizontal bars in the boxplots indicate the sample median, while the red horizontal bars indicate the sample mean.

It should be noted that the associated study of nitrogen isotope source tracking by Dr. Kiho Kim's group from American University found that the majority of Saipan shoreline surface water sites had $\delta^{15}N$ values greater than 3‰, suggesting the availability of sewage-derived N in the nearshore waters. Indeed, three clusters of sites along the western coast — around sites 8, 18 and 26 — had $\delta^{15}N$ values greater than 10‰, strongly suggesting that sewage derived N was the dominant source of N in the environment (Kim, 2019). In contrast, algae collected along the reefline had comparatively lower $\delta^{15}N$ values, indicating limited availability of sewage-derived N offshore. The availability of sewage-derived N was higher during March than in August (Kim, 2019). Radon measurements highlighted the spatially and temporally variability in the input of groundwater into the lagoon. As expected, groundwater inputs were higher during August (i.e., rainy season) and were the most pronounced near site 18. When surface and ground water were analyzed for nutrients, groundwater nitrate concentrations were nearly an order of magnitude higher than those in surface water, indicating that groundwater flow is an important pathway of nitrogen pollution (Kim, 2019).

The observations by the American University group for sewage-derived nitrogen, Radon patterns in surface and groundwater, and nutrients were complementary and consistent with our own microbiological source tracking observations regarding likely sources of sewage inputs to Saipan coastal waters. The whole region of the west central Saipan Lagoon shoreline appeared to be a relative "hot spot" for human FIB marker during the August 2018 sampling event. It was also a region of elevated human FIB maker during the earlier March 2018 sample event, but not to the same extreme degree as seen in the August 2018 samples. In general the region of the Saipan Lagoon from sample site S17 through S22 appears to be chronically elevated in human FIB marker over 100 tsc/100mL level for all the sample times observed (**Figure 9**). This likely represents a combination of non-point-source runoff and of groundwater discharge that has been contaminated by contact with human fecal material, perhaps indicating sanitary infrastructure problems (especially in the case of the groundwater contamination).

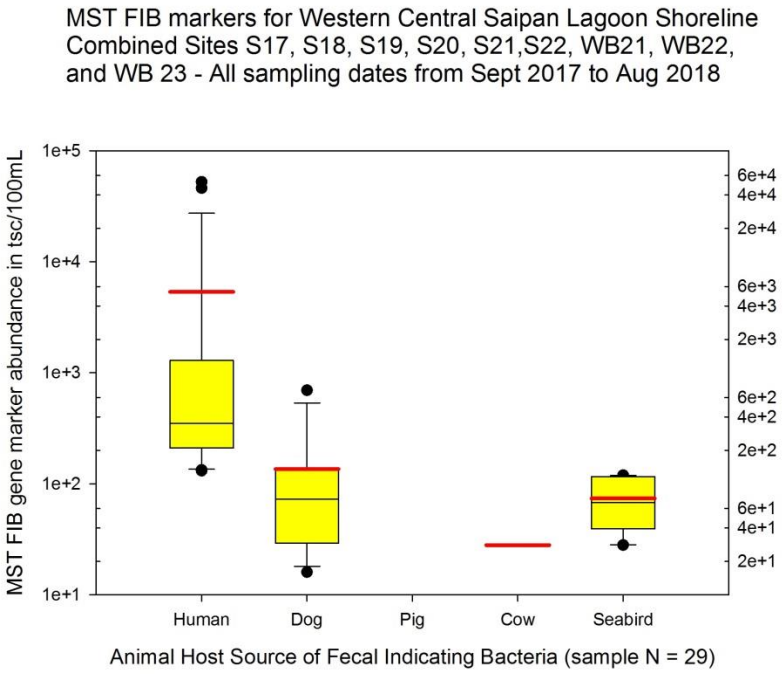


Figure 9: Boxplots summarizing the MST analysis for host-source FIB marker abundance for all of the water samples collected from the central shoreline of Saipan Lagoon where the highest human FIB marker exceedances were observed. The three highest outliers shown for the human marker boxplot represent groundwater samples taken from sites S18 and S19 (see Appendix 1 for specific values). Black horizontal bars indicate the median, and red horizontal bars indicate the mean.

Another area of chronic but highly variable elevation for human FIB marker was the "Grotto" site NEB01. Out of 6 samples collected from the Grotto during the three sampling events in Sept 2017, March 2018, and August 2018, 4 samples were over 100 tsc/100uL for human FIB marker, and 2 samples were over 1000 tsc/100mL (**Figure 10**). The highest levels were observed for samples collect on 3-16-2018 (at 3,003 tsc/100mL) and for samples collected on 9-13-2017. It is likely this highly variable but chronic appearance of human-source FIB marker may be due to direct shedding from high densities of recreational bathers and divers at this site. The Grotto is an extremely popular tourist destination, with a difficult and step stone stairway to the waters and the bottom of the Grotto and the only restroom facilities are at the top of the vehicle parking area of the Grotto, a difficult climb that many visiting swimmers may not be willing to make just for a use of a restroom facility. The random but high densities of visiting swimmers at this site most probably contributed to the pattern of FIBs that have been observed here. In addition, the high density crowds at the Grotto have been observed to potentially represent other health and safety risks, particularly during the ascent and descent of large crowds along the steep stairway leading from the parking area to the waters of the Grotto. More robust crowd control and/or limiting the bather density at any one time at the Grotto and/or allowing periods of low or closed activity at the Grotto to permit natural flushing of local waters might serve to improve both general safety and water quality of this popular and unique Saipan attraction.

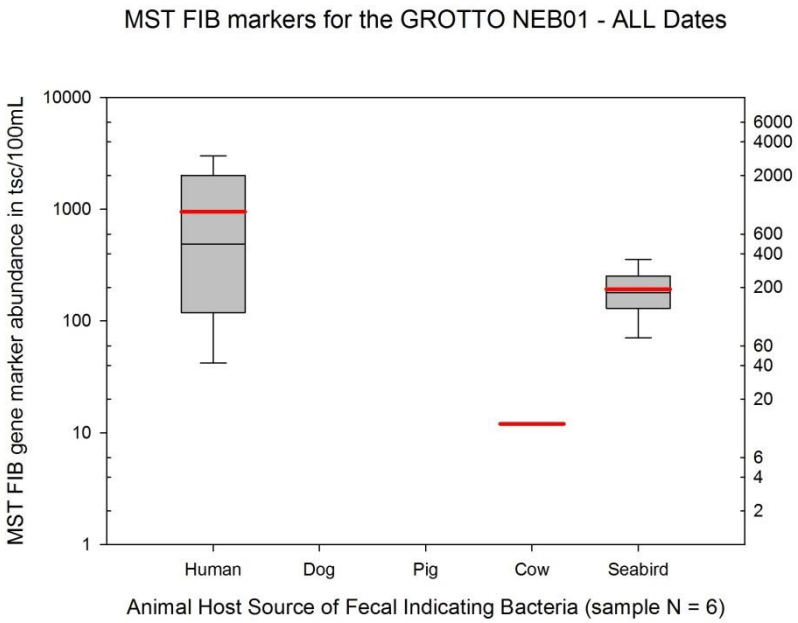


Figure 10: Boxplots summarizing the overall results from the Grotto for all sampling dates. Only human and bird markers were at significant levels. Black horizontal bars indicate the median, and red horizontal bars indicate the mean.

A number of other coastal shore sites have also shown relatively high levels of human Bacteroides FIB marker at various times during the study (see MST data table in **Appendix 1**), but there appear to be few consistent long-term patterns, other than chronic high levels along the shore of the central Saipan Lagoon. The area around the Garapan fishing dock seems chronically elevated, as does the drainage area of the Dai Ichi Hotel. However, a chronic enterococci hot spot at the American Memorial Park drainage does not appear to also be chronically elevated for human marker, even though it was frequently and significantly elevated for seagull/seabird marker. Other sites appear more temporally variable, for example the area around the Jeffery's Beach was very low for human FIB marker in March and August of 2018 even though there

were high levels of Enterococci, but there was elevated human FIB marker at Jeffery's Beach during the September 2017 sampling event. However, there were also frequently seabird FIB markers at Jeffery's Beach and it is likely that at least some of the observed Enterococci exceedances at this site may be due to bird fecal contamination.

Unsurprisingly, little human marker or agricultural marker was observed around Bird Island, and the predominant FIB marker was indeed for seagull/seabird.

Dog fecal bacterial marker appeared to be relatively wide-spread about the island, and many sample sites had significantly elevations of dog FIB marker, including sites like the Tanapag Meeting Hall area, the Hafa Adai and Dai Ichi hotels, the Central Repair Shop area, the Garapan Fishing Dock area, and in general throughout the western central region of the Saipan Lagoon shoreline.

Again no significant cow or pig associated FIB marker was observed anywhere during these studies, even though some roaming cattle were observed.

Regarding the offshore reef sites, significant levels of human-associate FIB genetic markers at levels over 100 tsc/100mL were found at 3 reef sites in March of 2018: at sites R06, R07, and R09, while in August of 2018 human FIB marker at levels over 100 tsc/100mL were found at reef sites R06 and R07. This indicates that LBSP derived microbial contaminants are indeed reaching the reef tract and that corals of this area of Saipan Lagoon are being exposed to these contaminants and therefore potentially exposed to pathogens and other contaminants that might be associated with this LBSP pollutant transport. Coral tissues were not tested during this particular study so it is not clear at this time if these microbial contaminants are being taken up into or influencing the coral holobionts at these reefs, but such impacts of LBSP microbial contaminants on coral microbiota have been documented at other coral reefs such as in southeast Florida (Staley et al, 2017). A follow up-study to actually test coral tissue along the reef tract for presence of fecal indicator markers would be useful to further define the reef exposures. From the pattern of results from this Saipan MST source tracking study, it would appear the most likely source of the exposure and impact to these reef sites is the western central Saipan Lagoon shoreline area, particularly in the region from roughly site S17 through S22 (i.e. roughly from the area of the Garapan Fishing Dock through to Susupe). Of particular note is the level of human-associated FIB genetic marker from area around sites S18 and S19 (particularly in groundwater) observed in August of 2018 that were orders of magnitude higher than had been observed earlier in the year during the March 2018 sampling event. These observations represented the highest levels of human fecal microbial contamination of the entire study. Due to the limited timeframe of the sampling, it is unclear if these extremely high levels were an isolated incident or reflective of broader chronic problems in this area, although elevated levels of human marker were observed in the region at all sampling time points. We suggest that this area warrants further investigation. It would be wise to conduct a more careful examination of the watershed for this region, particularly with an eye for improperly functioning or illicit sanitary infrastructure that

might be impacting ground water and shore water quality in this vicinity which in turn appears to represent a significant exposure source of microbial contaminants to the reefs of Saipan Lagoon.

CONCLUSIONS

- Both Human and Dog fecal bacterial indicators were found widespread throughout the coastal waters of Saipan, and both appear most frequently elevated in the western central shoreline region of Saipan Lagoon.

- Observations of elevated Human FIB marker are likely reflective of inputs from inadequate or improperly functioning sanitary infrastructure. This is especially likely in the area of the western coastline of the Saipan Lagoon. The results warrant further study of the temporal and spatial variability of human marker in groundwater, stormwater runoff, and surface waters, as well as an aggressive assessment of the current condition of sanitary infrastructure in the region.

- Some cases of isolated high temporal variability in human FIB marker (especially in remote but popular swim areas) may also reflect some degree of bather/swimmer shedding. Bather impacts may be a possible explanation for some chronic but highly variable FIB areas such as the Grotto, Jeffrey's Beach, Hidden Beach, etc. The Grotto especially is a high density bathing area with significant and frequent detection of human marker. While the Grotto clearly receives multiple inputs, the human input represents a substantial high-risk component of this. Further observations of the temporal variability of human marker in association with observations of human bather density at the Grotto are clearly warranted.

- Groundwater discharges into the western central region of Saipan Lagoon appear to have high levels of human fecal bacterial indicators, including some extremely high levels in the area ranging from sites S18 through S20. It is necessary to determine if these observations were isolated incidents during the period of this study or if they are representative of a larger chronic problem, thus a follow-up study of potential groundwater contamination is highly encouraged.

- Dog FIB levels were high at several regions around the island, especially around the Garapan area, the Tanapag area, and throughout the western central shoreline of the Saipan Lagoon. A better understanding of the temporal and spatial contributions of dog fecal contribution to regional water quality is needed, thus a follow-up study of this topic is highly encouraged.

- Birds would appear to be significant contributors of FIB to areas such as the American Memorial Park drainage, the Lau Lau Bay area, the Grotto area, Jeffry's Beach, Old Man by

the Sea, and of course Bird Island. Such bird fecal contamination is probably not manageable in a practical sense except in certain high-effort and expensive circumstances. However, the potential of bird fecal contributions and their relatively lower-risk should be considered when assessing observed exceedances of general fecal indicators such as Enterococci or E. coli, especially as bird fecal contributions may serve to confound general water quality assessments. If significant bird contribution is determined to be a primary contributing factor in recreational water sites of chronic exceedance without any other high-risk fecal input sources, then alternative site-specific water quality criteria may need to be considered. In this case, a combination of methods, including MST, would need to be deployed to prove that any such alternative criteria promulgated by the BECQ are equally protective of the public and environmental health.

- The greatest chronic export of high levels of fecal indicating bacteria, and particularly high-risk human-source fecal indicating bacteria, appears to be coming from the shoreline runoff discharge and the groundwater discharge of the western central Saipan Lagoon region stretching from roughly the Garapan Fishing Dock to Susupe, with the worst of the discharge from areas near sites S18 through S20. This is likely the LBSP microbial contaminant source causing the greatest risk exposure of pathogens to the coral reefs of Saipan Lagoon (at least during the time of this study) Reef waters offshore of this area showed the greatest abundance of LBSP microbial contaminants at the reef, particularly human FIB marker.

- This study demonstrates that MST can be an effective tool for helping to inform and enhance natural resource management for Saipan coastal waters by the BECQ. Maintaining and utilizing MST analytical capability may continue to help guide and inform management moving forward.

- We recommend follow-up source tracking studies to supplement the BECQ water quality surveillance program, including further investigation of groundwater, and we also suggest a further potential study examining actual coral tissue for evidence of LBSP microbial contaminant exposure to the coral reef holobiont communities.

Action Recommendations to BECQ:

- Further investigation of sanitary infrastructure and potential leakages especially in the region of the west central Saipan Lagoon shoreline area. Aggressively pursue the detection and repair of sanitary infrastructure in the region.

- Further investigation of potential groundwater contamination, particularly in the region of the west central Saipan Lagoon shoreline area. Groundwater testing during the current study was extremely limited and a larger and more systematic assessment of groundwater quality is essential.

- Further investigation of potential contamination of stormwater runoff, especially in the Tanapag region, Garapan district region (including American Memorial Park), and particularly the Susupe region, and the west central Saipan Lagoon shoreline region in general.

- Implementation of more robust control measures for dealing with animal waste, particularly dog fecal contamination, and especially along the western coast of Saipan Lagoon. A large number of dog fecal marker hotspots suggest that dog fecal influence is widespread. Among other things, consider mandating dog fecal cleanup policies, and provide resources to make such dog fecal cleanup in public spaces easy and cost-effective. We are not saying that dogs should be banned from beaches or recreational water spaces, however there are many good examples of effective dog-beach management practices. Still, it should be recognized that effective management of such animal waste needs to extend beyond just the beaches and be implemented throughout the watershed.

- Implementation of more robust bather crowd control measures at high density bathing beaches and recreational water areas, especially at the Grotto, along with more accurate bather density monitoring of crowds at the Grotto. Managing crowds at the Grotto to allow more time for better natural flushing between crowds might be advantageous to maintaining better water quality at the site, and would also likely provide a benefit in increased physical safety resulting from such crowd control.

- In addition to their routine water quality monitoring assessments, BECQ might consider also implementing a special study on the water quality impacts of tourism and how those impacts can be minimized and/or mitigated while still maintaining a healthy and active tourism industry. Along with this, a public education campaign on beach and recreational water hygene (particularly for tourist populations) could be useful to better inform the public of both the risks and health benfits of using Saipan's natural recreational water resources, and how they can minimize the risks from recreational water exposure while maximizing the health benefits of such exposure. The public should be highly encourage to continue to utilize these "blue gym" resources as much as possible since the health benefits are extensive and undeniable, but their personal behavior and hygene at the beach or the Grotto can have an impact on their personal health risk exposure and the area's water quality.

- Integration of periodic microbial source tracking assessments into the more routine water quality monitoring efforts of BECQ would permit a better understanding of the seasonality, infrastructure, environmental and social factors influencing source contributions to local waters. Consider future special MST studies or perhaps quarterly MST assessments to complement regular ongoing monthly water quality monitoring operations.

- Given the fact that fecal detection of cow and pig livestock contamination was much lower than originally expected, further testing of the local detection sensitivity levels for the CowM2 qPCR assay and the Pig2Bact qPCR assay for known feces dilution concentrations of local Saipan livestock populations could be useful to determine if there are any local or regional factors (livestock diet, etc.) that might affect the local detection sensitivity of these two livestock assays in Saipan specific samples. If this turns out to be the case, then modified protocols could be developed in collaboration with BECQ to boost detection sensitivity for local livestock fecal contamination.

- Additional and on-going training for current and future BECQ workforce in molecular water quality assessment techniques needs to be considered and planned for to maintain an enhanced BECQ analytical capability for environmental water quality assessments. A training and proficieny validation plan should be developed and implemented for the BECQ workforce employed in such water quality assessments with appropriate resources for maintaining future proficiency of new analytical staff.

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