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Consideration of Natural Sources in a Bacteria TMDL—Lines of Evidence, Including Beach Microbial Source Tracking

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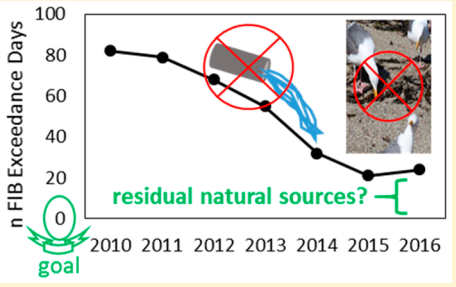
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Supporting Information

ABSTRACT: Total Maximum Daily Load (TMDL) stipulations remained unmet at a southern California beach despite a suite of management actions carried out since 2001, prompting exploration of a Natural Sources Exclusion (NSE) provision within the TMDL. Quantitative Microbial Source Tracking (MST) was employed from 2012 to 2015 to inventory sources of natural and anthropogenic fecal indicator bacteria (FIB). Data suggested FIB exceedances could be traced to gulls based on gull marker prevalence and correlations with FIB concentrations in seawater, sand, and eelgrass. In contrast, human marker concentrations and a tracer dye test did not indicate prevalent human sources. Exponential decay of gull marker in sand amended with live *Catellicoccus marimammalium* suggested that measured marker reflected fecal inputs versus growth outside the host. Improved water quality was coincident with a 2013 bird exclusion structure, consistent with NSE. However, load allocation needed for TMDL reconsideration was hampered by variable ratios of FIB, MST markers, and pathogens measured in seawater and in gull, cat, and raccoon feces. Quantitative Microbial Risk Assessment is a suggested path forward because such models can incorporate distributions from a combination of FIB sources and communicate criteria in terms of human health risk.



INTRODUCTION

Contaminated water negatively affects coastal economies and human and ecosystem health. Total Maximum Daily Load (TMDL) implementation plans are a regulatory consequence for waters that fail to meet water quality standards. TMDL implementation to remediate impaired water quality is a multibillion dollar problem for the U.S. annually,[1] and TMDLs aimed at improving bacterial water quality are among the most common. For example, 56% of the coastal shoreline was listed as impaired for bacteria in California in 2012,[2] and the majority of listed water quality impairments were from unknown or nonpoint sources. Identifying the specific source of microbial pollution is difficult in part because the fecal indicator bacteria (FIB) used to regulate water quality are not host specific. *Enterococcus* spp. (ENT), *Escherichia coli* (EC), fecal coliform (FC), and total coliform (TC) are present in the gut microbiomes of a variety of animals and are found in nonfecal sources, such as sand or vegetation.[3]

Lack of host specificity and survival of FIB outside the host and can uncouple FIB from the pathogen loads for which they are meant to proxy.[4,5] Measured bacterial concentrations can overestimate risk relative to regulations, which assume that FIB originate from human fecal sources.[6,7] Some TMDLs recognize the possibility of decreased risk and provide a Natural Sources Exclusion (NSE) approach to reconsider TMDL criteria.[8] Microbial Source Tracking (MST) and Quantitative Microbial Risk Assessment (QMRA) are tools that can aid evaluation.[9] Quantitative MST employs qPCR to amplify marker genes associated with the gut microbiomes of specific animals (e.g., humans, birds, dogs). Identifying the host source of fecal contamination provides opportunities to devise effective management action.[10] MST also can be utilized in QMRA models.[6,7] QMRA estimates human health risk based on pathogen concentrations associated with a particular fecal source. The results can help managers prioritize areas in greatest need of remediation or, alternatively, develop specific criteria for sites with less risk.[6,7]

Inner Cabrillo Beach (ICB) located in Los Angeles County, California (Figure 1, Figure S1 of the Supporting Information, SI) is an example of a site with a TMDL that includes an NSE provision. This TMDL, along with others in the region, interpret load allocations based on federal guidelines,[11] additional state-specific criteria,[12] and comparisons to a reference beach.[9] Load allocations are expressed in terms of the allowable number of days bacterial concentrations may exceed numeric criteria for single samples and for the geometric mean of the samples.[13,14] For instance, no "exceedance days"

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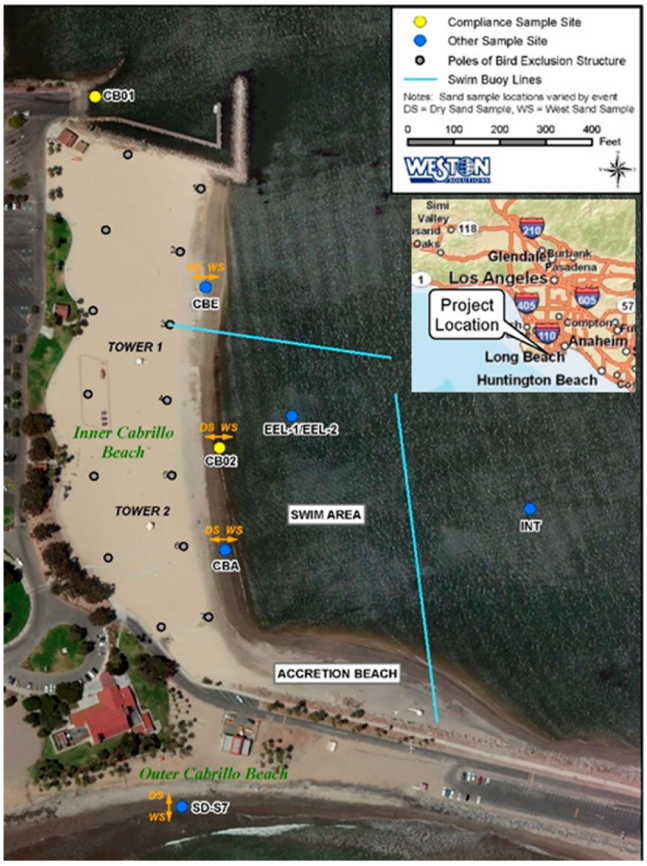


Figure 1. Sampling station locations. Outer Cabrillo Beach (OCB) is open-wave and Inner Cabrillo Beach (ICB) is semienclosed (see Table S2 for details). Bird exclusion structure shown prior to extension. Located in Los Angeles, CA. Imagery source: Esri, DigitalGlobe, GeoEye, i-cubed, USDA, USGS, AEX, Getmapping, Aerogirid, IGN, IGP, swisstopop, and the GIS User Community.

are allowed at any time for the FIB geometric mean (35, 200, and 1000 MPN/100 mL for ENT, FC, and TC, respectively) or for the single sample maxima (104, 400, and 10 000 MPN/100 mL for ENT, FC, and TC, respectively) in summer dry conditions (April 1—October 31).[13] The NSE provision offers the ability to reconsider the number of allowable exceedance days if the reference beach is not suitable[8,9] (such as for semienclosed beach sites), anthropogenic sources of FIB are controlled, and natural sources such as birds are quantified.[8,13]

ICB is a small sand beach (~335m) adjacent to a quiescent swim area located within an urbanized watershed. The beach area is historic with a 1930s landmark located on Outer Cabrillo Beach (OCB) (Figure 1). Cabrillo Marsh (Figure S1), a restored tidal marsh located north of the beach, represents the remaining area of historic marsh land.[15] The area has undergone major structural repairs and a suite of management actions totaling over $23 million since 2001 to control sources of FIB, with details provided in the SI. Despite management action, FIB concentrations continued to exceed numerical water quality objectives stipulated in the TMDL. Exploration of the NSE provision was motivated by continued water quality exceedances in the context of site conditions (semienclosed, frequented by hundreds of birds daily,[16] daily transport of water[17] from adjacent eelgrass beds[18]).

As one of the first formal NSE investigations, work at ICB proceeded iteratively under the guidance of the Los Angeles Regional Water Quality Board in an approach consistent with the TMDL language,[8,13] the California Microbial Source Identification Manual,[19] and the QMRA framework provided by the U.S. Environmental Protection Agency.[20] FIB sources were evaluated with multiple lines of evidence that included historical review; testing sanitary infrastructure; coupled FIB-MST quantification in seawater, sand, sediment, and eelgrass; and evaluation of the efficacy of management action to control bird populations at the beach. In addition, preliminary analysis of pathogens in gull, cat, and raccoon feces was performed to inform risk analysis. Overall, this work evaluated bacteria sources in the context of TMDL stipulations and within the NSE provision, in particular.

MATERIALS AND METHODS

Sample Collection. Thirteen surveys in the period 2012—2015 were conducted. Sampling commenced at ICB 15 to 30 min following a moderate high tide in morning hours when prevailing off-shore winds tended to be calm (Table S1). Outer Cabrillo Beach is considered an open-wave location whereas Inner Cabrillo Beach is considered semienclosed (Figure 1). Beach swash zone stations included a TMDL compliance monitoring point at ICB (CB02), historical stations along the ICB beach length (CBA and CBE), and a compliance point at OCB (SDS7), which was under a separate TMDL (Table S2). Off-shore sites included a station outside the swim zone (INT) and stations located more nearshore within the swim area. For the later sites, samples were collected either without disturbing the bottom (EEL1) or with suspended eelgrass bed sediment (EEL2) (Table S2). An additional compliance monitoring point (CB01) was located at a boat ramp near a small wetland; the location was separated from ICB by a breakwater (Figure 1).

A variety of sample matrices were sampled in addition to seawater. Sample media included wet sand collected adjacent to CB02, CBA, CBE, and SDS7; dry sand collected in line with CB02 and SDS7; eelgrass bed sediment from EEL2; eelgrass wrack from the vicinity of CB02; and fresh eelgrass from the vicinity of EEL2 (Figure 1). Wet sand was collected from the swash zone; dry sand was collected approximately 10 feet above the highest swash zone observed for the day. The following number of samples were collected during these surveys for analysis by culture and molecular methods: seawater n = 331, wet sand n = 120, dry sand n = 30, sediment n = 25, fresh eelgrass n = 26, and wrack n = 17.

The City of Los Angeles Bureau of Sanitation Environmental Monitoring Division ("City of LA") provided membrane filters (as described in the SI for molecular analysis) for seawater filtered from station CB02 on days in which FIB concentrations exceeded water quality objectives during routine monitoring for FIB compliance in 2015 (termed "exceedance filters", n = 11). Data from FIB compliance monitoring at station CB02 (2010—2016) also were analyzed (see SI for monitoring schedule and dates).

As outlined in the SI, samples of seawater (n = 30, 30 separate days) and gull feces (n = 30, 6 separate days) were collected for pathogen and FIB analysis. Samples of cat (n = 15, 2 separate days) and raccoon feces (n = 17, 1 day) were obtained from in or near the marsh (Figure S1). Sample age varied but was not known.

Sample Analysis for FIB and MST. The SI provides details about sample collection and processing for seawater, sand, eelgrass (fresh and wrack), and feces. Details regarding

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culture and qPCR analysis include information on controls (including inhibition results), qPCR primers/probes, and references (Table S3). Briefly, samples were analyzed by culture for enterococci (cENT), *E. coli* (EC), and total coliform (TC). Samples were analyzed by qPCR for enterococci (ENTqPCR) and for markers associated with the gut microbiomes of human (HumMST), gull (GullMST), and canine (DogMST) hosts (Table S3). In addition to concentration data, qPCR values were compared to standard curve metrics (Table S4) in terms of copies per reaction (cpr) to determine if values were within Range of Quantification (ROQ), Detectable but Not Quantifiable (DNQ), Below Detection Limit (BDL), or nondetect (ND). Below Limit of Quantification (BLOQ) = DNQ + BDL. Detection frequencies were reported in terms of %pres = 100−%ND (Tables 1 and S5). All replicates were used to generate sample concentrations, with nondetects substituted as described in the SI. All sample concentrations were used to generate site calculations, with recognition that the confidence interval of BDL values may include zero and that inclusion of BDL values in the detection frequency can decrease assay specificity.[21−23] See SI for further discussion.

Table 1. MST and Enterococci Prevalence and Abundance in Seawater and Wet Sand for Semienclosed versus Open-Wave Stations[a]

	MST				FIB			
	human		gull		ENTqPCR		cENT	
seawater	ICB	OCB	ICB	OCB	ICB	OCB	ICB	OCB
geomean	36	57	4975	445	5392	546	35	8
%pres	4	19	96	56	100	100	79	29
wet sand	ICB	OCB	ICB	OCB	ICB	OCB	ICB	OCB
geomean	3.4	49	1140	39	418	17	3	1
%pres	0	0	94	54	100	87	80	7

[a]ICB = semienclosed Inner Cabrillo Beach stations CB02, CBE, and CBA combined; OCB = open-wave Outer Cabrillo Beach station SDS7; sea = seawater; sand = wet sand; %pres = 100−%ND, where ND = not detected. Units for cENT are MPN/100 mL or MPN/g dry, otherwise in copies/100 mL or copies/g dry. See Table S5 for all stations and matrices and additional descriptive statistics.

Sample Analysis for Pathogens. In addition to FIB and MST analysis, samples of gull feces and seawater were analyzed for the bacterial pathogens *Salmonella* spp. and *Campylobacter* spp. by a most probable number (MPN) method using culture on selective media for identification and DNA sequencing for verification. Samples of cat feces were analyzed for the protozoan *Toxoplasma gondii* and raccoon feces for the roundworm *Baylisascaris procyonis* using microscopic and PCR methods with verification by DNA sequencing, in addition to FIB and MST analysis. Details and references are provided in the SI.

Gull Marker Persistence Study. To enhance the interpretation of MST results in the context of gull marker prevalence, *C. marimammalium* culture was amended to laboratory mesocosms containing moist, aged sand. The concentration of GullMST was analyzed over a 28-day period (n = 24) to assess potential for bacterial growth. Mesocosms were incubated in the dark under humid conditions at 22 ± 2 °C; see SI for details.

BMP Evaluation. During the course of this study, an existing bird exclusion structure that covered ~2/3 of the sandy area (upper beach to just above the high tide line, + 8 ft MLLW)[16] was extended into the swash zone (0 ft MLLW) (Figure S1). This BMP was constructed between April 5—August 13, 2013, which included Surveys 6 and 7 (Table S1). BMP efficacy was evaluated by comparing FIB, ENTqPCR, GullMST, and HumMST results before and after construction

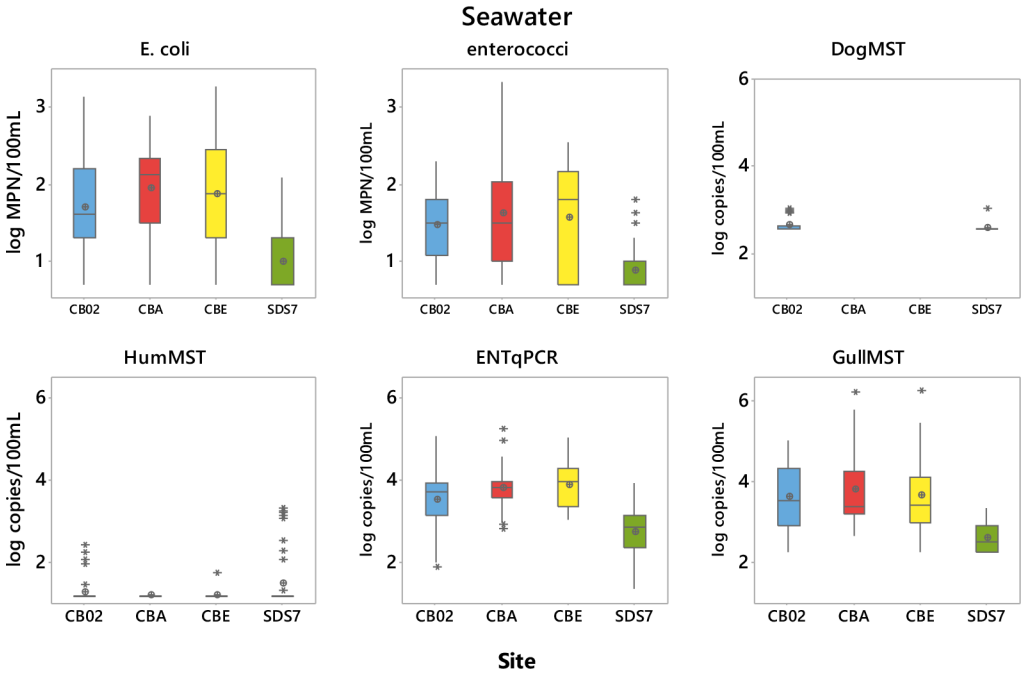


Figure 2. Concentrations of FIB and MST markers in seawater from ICB stations (CB02, CBA, CBE) compared to the open-wave beach station (SDS7). FIB were measured by culture (MPN/100 mL) and by qPCR for enterococci (ENTqPCR, copies/100 mL). Samples were collected during special study surveys (Table S5). Box and whisker plots show quartiles (25th and 75th percentile), median (horizontal line), and mean (circle with cross hair).

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for time periods that varied by rainfall and season, as detailed below. Samples were collected from station CB02 during FIB compliance monitoring (see SI) and special study surveys (Table S1).

Statistical Analysis. ANOVA hypothesis testing (α = 0.05), Spearman rho correlations, and nonparametric statistical analysis were performed using NADA[24] macros for censored data (Kruskal–Wallis = censKW.mac v.3.4, α = 0.05) using Minitab16 software. Simple calculations were performed without consideration to weekly or "rolling" permutations.[25] Data officially reported for bacterial compliance monitoring are available elsewhere.[15]

Sanitary Sewer Assessment. Beach bathrooms were dye tested using eosine (EOS) or rhodamine WT (RWT). Groundwater wells (Figure 1B) were monitored using charcoal packets (n = 10 pre, 47 post dye release) and seawater was monitored on-site for rhodamine using a YSI Model 6600 data sonde. Groundwater and surf zone samples were analyzed in the laboratory by spectrofluorophotometer for EOS and RWT (n = 10 pre, 88 post). Sand samples collected during well installation were analyzed for HumMST. See SI for details.

RESULTS

FIB and MST. To help identify FIB sources at ICB, MST markers for humans, gulls, and dogs were analyzed in seawater, sand (wet and dry), eelgrass (fresh and wrack), and sediment from the eelgrass bed (Figures 1 and S1). Factors known to affect bacterial concentrations include rain, tide, solar radiation, wave dilution, and wind.[26] Site conditions (Table S1) and historical documentation, including hydrologic characteristics, are provided in the SI.

Seawater Results. Gull marker and ENTqPCR were prevalent and abundant compared to human or dog marker in seawater samples collected during the 13 special study surveys (Table 1, Figure 2). GullMST amplified in the majority of DNA extracts from seawater samples collected from ICB, with only 4% nondetects (Table S5). Conversely, 96% of those same samples were nondetects for human marker. No HumMST values were in the ROQ, and all but one of the BLOQ results were below the LOD; numeric values (Table S5) primarily reflect the substitution value for nondetects. No samples exceeded the LOD for the dog marker (Table S5), although that assay was less sensitive than HumMST (Table S4). In contrast, enterococci amplified almost exclusively in the ROQ with only 1% nondetects (Table S5). Highest concentrations also were observed for gull marker and ENTqPCR on days with FIB exceedance (single sample criteria exceeded for *E. coli* = 8 days; enterococci = 17 days). Samples were collected during special study surveys (Table S5) and compliance monitoring (Table S6), and all samples collected that day were used in the analysis (Figure 3; n = 26 *E. coli* samples; n = 50 enterococci samples; no samples exceeded TC criteria).

Concentrations of FIB and gull marker were significantly higher in seawater from ICB compared to the open-wave station located at OCB (α = 0.05). Average GullMST concentrations at ICB were 87 times higher than OCB. HumMST was the exception to this pattern of higher concentrations measured at the semienclosed location; instead higher concentrations and more detects were measured at the open-wave site (Table 1).

A general cross-shore trend was observed with highest FIB concentrations in the ICB swash zone (Figure S2), supporting

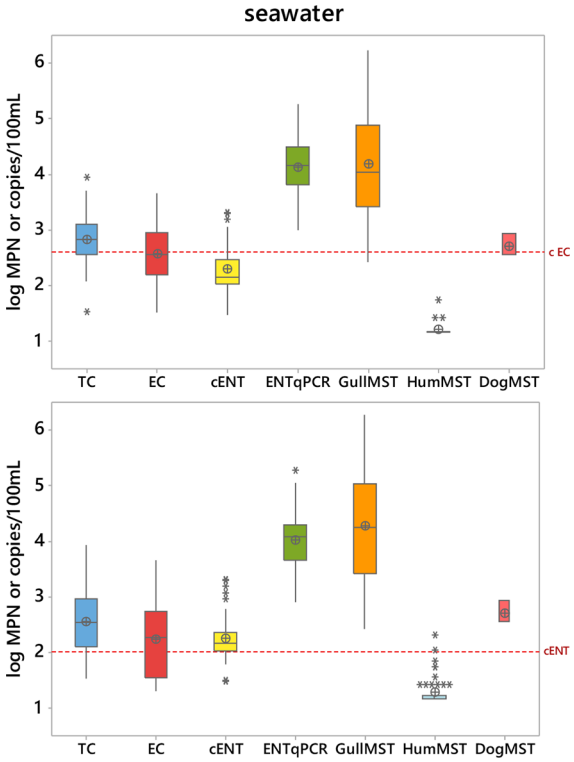


Figure 3. Concentrations of FIB and MST markers in seawater from enclosed shoreline stations (CB02, CBA, CBE, or CB01). FIB were measured by culture (MPN/100 mL) and by qPCR for enterococci (ENTqPCR, copies/100 mL). Samples were collected during special study surveys (Table S5) and compliance monitoring (Table S6) on a day in which at least one sample exceeded the single sample criteria (red line) for either *E. coli* (top, n = 26) or enterococci (bottom, n = 50). All samples collected on the day of exceedance are included. Plots as described in Figure 2.

the presumption of a shoreward source of bacteria versus transport to the beach from an off-shore point source.[15] Average swash zone concentrations of FIB from the three ICB shoreline stations did not vary significantly for the combined survey data (CB02, CBA, CBE; α = 0.05; Figure 2). Seawater concentrations of FIB determined by culture and qPCR were significantly higher at ICB compared to outside the swim area (INT) (α = 0.05). However, differences between the eelgrass bed and the CB02 swash zone were not significant. The highest individual concentrations of ENTqPCR, EC, and TC were measured in samples taken from near the bottom of the eelgrass bed, implicating the bed as a natural source of FIB to the overlying water column.

Sand, Eelgrass, and Sediment Results. In wet and dry sand, concentrations of FIB, ENTqPCR, and GullMST were higher from ICB compared to the open-wave beach (α = 0.05). HumMST did not amplify in any sand samples from either location (ND = 100%). In contrast, GullMST and ENTqPCR were prevalent and abundant in both wet and dry sands at ICB, with the majority amplifying in the ROQ (Table S5). DogMST did not amplify in any sand samples from SDS7, any dry sand samples from ICB, nor the majority of wet sand samples from ICB (Figure S5). Enterococci concentrations both by qPCR and by culture were high in eelgrass wrack and in fresh eelgrass compared to wet and dry sand (Figures S6 and S7). High GullMST marker concentrations also were observed in these matrices. The exception to this pattern was sediment from the

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eelgrass bed which showed high ENTqPCR concentrations but relatively low GullMST concentrations (Figure S9).

Correlations between wet sand and seawater were observed for all cultured FIB, gull marker, and enterococci by qPCR at ICB. DogMST and HumMST were the exceptions to this coupled wet sand−seawater pattern (all wet sand HumMST were nondetects). Additionally, strong correlations were measured in seawater between FIB, GullMST, and ENTqPCR (Tables 2 and S7). GullMST in eelgrass wrack was correlated to ENTqPCR in wrack and to ENTqPCR in seawater (spearman rho = 0.85 and 0.76 for wrack−wrack and wrack−seawater, respectively; p-value <0.001).

Table 2. Enterococci and MST Correlations for ICB Seawater and Wet Sand[a]

parameter	n	GullMST, seawater
GullMST, seawater	85	
GullMST, wet sand	85	0.77
ENTqPCR, seawater	85	0.84
ENTqPCR, wet sand	85	0.73
cENT, seawater	80	0.66
cENT, wet sand	80	0.56
DogMST, seawater	24	NS
DogMST, wet sand	30	NS
HumMST, seawater	85	NS
HumMST, wet sand	45	NS

[a]Spearman Rho correlation coefficients; NS = not significant at α = 0.005. See Table S7 for additional correlations and details.

Pathogens, FIB, and MST. *Seawater Results.* Seawater was analyzed for *Salmonella* and *Campylobacter* spp. with results of method validation provided in the SI. Pathogen concentrations in seawater were predominately nondetects (<0.65 MPN/100 mL) with no pattern observed between indicators and pathogens; MST markers were not analyzed for these samples (Tables 3 and S8). FIB concentrations mostly met water quality objectives at the time pathogen samples were collected. On the occasions of ENT exceedance (3/30 samples >104 MPN/100 mL), neither *Salmonella* nor *Campylobacter* spp. were detected. Conversely, FIB concentrations were at or below the level of detection on the two instances in which *Campylobacter* spp. were detected.

Feces Results. The pathogens *Campylobacter* and *Salmonella* spp. were each detected in 40% of gull fecal samples tested, with at least one of these pathogens in 73% of the samples (22/30) at concentrations that ranged from <1.8 to >1600 MPN/g wet (Table S9). Both pathogens were detected in two samples at low to intermediate concentrations (1.8—19 MPN/g wet *Campylobacter* and 2—21 MPN/g wet *Salmonella*). All MPN culture confirmations were further verified by sequencing (GenBank accession numbers KY767034 - KY767482). All *Salmonella* spp. were BLAST-identified as *Salmonella enterica* (n = 99 with 100% sequence identity; n = 2 at 99%). Campylobacter sequence identification was more varied; out of 114 sequences, *C. jejuni* (n = 55), *C. coli* (n = 5), and *C. lari* (n = 15) were identified at 100% sequence identity. Other sequences were annotated as *C. jejuni*, *C. coli*, *C. lari*, *C. insulaenigrae*, or *C. subantarticus* (n = 31, 99% match), *C. lari*, *C. volucris*, *C. subantarcticus*, or *C. peloridis* (n = 2, 99% match), and six sequences were identified only to the genus level.

In gull feces, concentrations and ratios of all measurements varied highly. Other than a correlation between *E. coli* and total coliform, no pattern was evident between FIB, MST markers, or pathogens in gull feces (Table S10). Gull marker was typically, but not always, higher than enterococci (median GullMST:ENTqPCR = 63, range = 0.3—5627), and the pattern of gull marker to enterococci ratio observed in DNA extracts did not always match the pattern in paired samples analyzed for cultured enterococci (Table S10). The majority of gull fecal samples amplified in the ROQ for GullMST (14/18) and ENTqPCR (17/20), with no amplification of HumMST (100% ND), with the caveat that five samples showed PCR inhibition that was not fully resolved by DNA dilution (see SI). Pathogens were not detected in the samples with the highest gull marker concentrations (samples 22 and 25) (Tables S9 and S10).

The protozoan *T. gondii* was not observed in cat fecal samples by microscopic or PCR analysis (Table S11). The raccoon roundworm *Baylisascaris* spp. was identified in all raccoon fecal samples by microscopy, with verification in the majority of samples via PCR and amplicon sequencing (Table S12). Enterococci concentrations varied and were markedly higher in raccoon than in cat or gull feces (up to 6500 times higher) (Tables S11, S12, and S13). No pattern was observed between enterococci concentrations and *Baylisascaris* spp. egg

Table 3. Concentrations of Enterococci, Human Marker, and Pathogens in Seawater and Fecal Samples[a]

parameter	FIB		MST	pathogen	
seawater	**cENT**	**ENTqPCR**	**HumMST**	***Campylobacter***	***Salmonella***
geomean	15	nts	nts	0.09	<0.33
%pres	70			7	0
feces					
gull	**cENT**	**ENTqPCR**	**HumMST**	***Campylobacter***	***Salmonella***
geomean	3.5×10^6	6.5×10^4	<615	8	5
%pres	100	100	0	40	40
cat	**cENT**	**ENTqPCR**	**HumMST**	***T. gondii***	
geomean	1.8×10^7	2.7×10^6	707	ND	
%pres	100	100	20	0	
raccoon	**cENT**	**ENTqPCR**	**HumMST**	***Baylisascaris***	
geomean	2.1×10^9	4.2×10^8	2092	187	
%pres	100	100	40	100	

[a]Units are MPN or copies/g wet for feces. %pres = 100−%ND; nts = not sampled on day of pathogen collection. See the SI for GullMST results and other details: seawater−Table S8; gull feces−Table S9; cat feces−Table S10; raccoon feces−Table S11; and summary statistics−Table S12.

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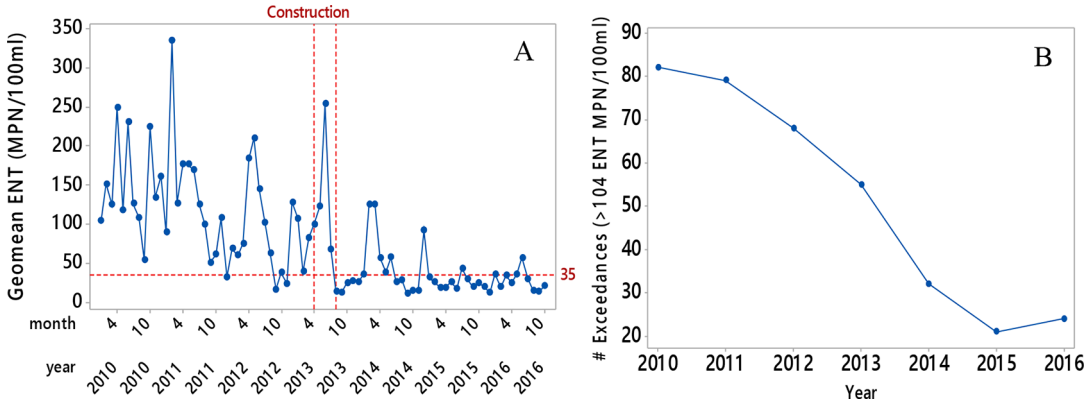


Figure 4. Decline over time at ICB for (A) the enterococci geomean calculated by calendar month for all months and weather conditions; "construction" shows period of construction of the extended bird exclusion structure and (B) number of single sample maximum exceedances for the time period April 1—October 31 of each year.

Table 4. Seawater FIB and MST Before and After Bird Exclusion Structure Extension[a]

cumulative rain (in)[b]		parameter	geomean		n exceedance days, ENT		dates n days	
before	after		before	after	before	after	before	after
Compliance monitoring								
			summer & winter				8/8/2010-3/30/2013	8/20/2013-3/26/2016
			91	**28**	315	**133**	679	679
31.9	23.9	**cENT**	wet weather removed					
			88	**25**	141	**52**	327	327
			summer only				4/1/2012-10/31/2012	4/1/2013-10/31/2014
			81	**24**	68	**21**	154	154
1.7	3.0	**cENT**	wet weather removed					
			81	**24**	60	**17**	132	132
			winter only				11/1/2012-3/31/2013	11/1/2013-3/31/2014
			64	51	39	37	108	107
6.3	4.1	**cENT**	wet weather removed					
			44	44	17	27	68	87
Special study surveys[d]								
			summer & winter, no wet weather				8/6/2012-3/25/2012	9/5/2013-1/6/2015
		cENT	40	**8**				
		cEC	39	**14**				
6.4	10.6	**cTC**	221	**99**			5	6
		ENTqPCR	7540	**470**				
		GullMST	3017	**1323**				
		HumMST	<26	41				

[a]Samples collected from the swash zone at station CB02. Bold indicates significant decline in geomean or exceedance days "after" extension of structure (α = 0.05), except for GullMST which was significant at α = 0.1 for geomean values and α = 0.05 for average values (8286 before, 2456 after). [b]Rain at Los Angeles airport from http://www.cnrfc.noaa.gov/monthly_precip_2015.php. [c]Conditions as per regulation: Summer: April 1—October 31, Winter: November 1—March 31; "remove wet" = data removed for wet weather days, defined as ≥0.1 in. of rain and 3 days after.[12,13] [d]For special study surveys (Table S1), multiple samples were collected per day resulting in samples before: n = 27; samples after: n = 15 for MST, n = 8 for FIB. Units of cENT, cEC, cTC in MPN/100 mL; ENTqPCR, GullMST, HumMST in copies/100 mL; insufficient sample size to compare DogMST.

counts in the samples of raccoon feces. Human marker showed amplification with 40% (6/15) of raccoon fecal samples (814—94,838 copies/g wet), some cat feces (3/15, BDL), and no gull fecal samples (0/18) (Table S10).

Persistence of Gull Marker. Persistence and regrowth in environmental matrices complicates interpretation of FIB and MST data,[27] especially if the bacteria targeted by MST also persist or grow in the environmental matrices studied. To our knowledge this is the first report of decay rates based on amendment with live *C. marimammalium* culture. Growth of *C. marimammalium* was not observed in sand microcosms designed to favor persistence (moist, dark, and sand aged to remove predators). Exponential decay of GullMST was observed over the course of the study (k = 0.30 d^{-1}) which translated to a half-life ($t_{1/2}$) of 2.3 days and a t_{90} of 7.7 days. The initial amendment provided a GullMST concentration of $3.67 \times 10^6 \pm 6.25 \times 10^5$ copies/g dry (average ± stdev). At day 28, the concentration remained in the ROQ (1556 ± 1124 copies/g dry; LOD was 8 cpr for this experiment, Table S4). The initial spike was higher than measured in ICB sand (geomean (range): 1140 (19−21 498) copies/g dry, n = 85 wet sand; 1410 (178−14 265) copies/g dry, n = 14 dry sand). On the basis of the wet sand geomean and maximum concentrations, detectable signal could have persisted for 9 to 19 days under these experimental conditions at observed initial concentrations. Observed persistence was similar to human

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MST marker incubated under dark conditions in other studies (T_{90} = 8.7 d); however, more rapid decay is expected under natural conditions. For a different *C. marimammalium* marker (CAT), more rapid decay was observed in feces-amended microcosm studies under natural sunlight and predation conditions (T_{90} values ~1 day or less).[28]

Bird Exclusion Structure Effect. During the course of this study an existing bird exclusion structure was extended into the swash zone (Figure S1). The initial structure, installed in July 2010, appeared to affect bird behavior based on bird counts conducted by the Cabrillo Marine Aquarium (n = 63 surveys, July—December 2010). Fewer birds were observed on ICB sand directly under the structure (297 under versus 1493 outside the structure), and less birds were observed on the ICB beach face versus on the accretion beach (see Figures 1 and S1), where no structure was present (2,008 birds at ICB versus 9035 accretion beach). In the study here, downward trends in FIB concentrations and in the number of single sample exceedances were apparent since 2010 (Figure 4). There was concern, however, that below average rainfall in the region[29] contributed to recent improved water quality rather than BMP efficacy. A variety of comparisons were made before and after the structure to capture a mix of seasons, weather conditions, and sample sizes. For compliance monitoring, only enterococci data were evaluated because it tends to dominate exceedances.[15] All three FIB were evaluated for the data generated in the special study surveys described here.

In general, the extension of the bird exclusion structure coincided with significantly improved water quality as measured by the number of ENT exceedance days and concentrations of cultured FIB, enterococci by qPCR, and gull marker (Table 4, α = 0.05). Out of the parameters analyzed, only HumMST failed to show a decrease in concentration after completion of the structure (Table 4). For three out of the four time periods evaluated, cENT declined significantly (α = 0.05), even with wet weather measurements included and with higher monthly rainfall after structure completion. The exception to significantly improved water quality after structure completion was an evaluation period that was drier after construction (winter condition measurements only, Table 4). These results suggest that decreased precipitation alone was unlikely to account for the declines observed in Figure 4. This supposition is consistent with a lack of stormwater inputs to ICB; diversions are in place as part of prior BMPs (see SI). However, it is not possible to entirely rule out drought effects given that groundwater flows were likely altered due to the severity of drought conditions.

Sanitary Sewer Assessment Results. MST was coupled with a tracer dye test to investigate possible sources of anthropogenic FIB. This evaluation did not reveal leakage from beach bathrooms based on eosin or rhodamine measured in groundwater (n = 57) and swash zone samples (n = 88 each for spectrophotometer and for sonde) (Table S14). HumMST was not detected in groundwater-saturated sand samples collected during installation of beach wells (Figure S1) and reactions passed qPCR inhibition control analysis (n = 10).

DISCUSSION

Like many beaches, ICB is a nonpoint source beach with a mixture of bacterial sources. Quantitative MST was employed to help inventory natural and anthropogenic sources of FIB. Data were consistent with an NSE approach, which requires predominately natural sources, such as birds.[13] Results suggested that FIB at ICB could be traced to gull populations (Table 1), even on days in which samples failed to meet water quality criteria (Figure 3). The highest concentrations were measured for gull marker and enterococci by qPCR, with the same samples showing less dog marker and relatively little human marker (GullMST > ENTqPCR > DogMST ≫ HumMST in DNA extracts; GullMST average concentrations >1000 times higher than HumMST; Table 1, Table S5). An interconnection between FIB and gulls at ICB was observed across every matrix except eelgrass bed sediment, which instead showed high concentrations of ENTqPCR against relatively low GullMST and cENT (Table S5). No significant correlations of FIB with human marker were observed at any station (Table 2, Table S7), and the dye tracer study was negative (Table S14). Consistent with the MST results, extension of the bird exclusion structure was associated with improved water quality at ICB (Table 4, Figure 4).

Prior library-dependent and enterococci speciation studies (see SI) suggested that sand and eelgrass could be important natural sources of FIB to ICB. A number of conceptual models demonstrate how environmental substrates can act as bacterial reservoirs with accumulation in the swash zone through deposition and resuspension cycles,[30] particularly in low energy regimes.[31] This study supports those ideas, with evidence seen in FIB concentration correlations, particularly between seawater and wet sand (Tables 2, S7). Measurement of enterococci by qPCR helped clarify these patterns versus culture analysis alone.

Although multiple lines of evidence suggested that natural sources predominated at this site, a TMDL reconsideration through a NSE approach calls for load allocation of nonpoint sources. This is difficult because MST lacks markers to identify nonfecal sources, such as naturalized FIB in sand or vegetation,[32,33] and concentrations of markers do not directly translate into concentrations of FIB. Correlations between FIB and GullMST were observed for seawater and wet sand (Table 2), but ratios varied widely in feces (Table S10) confounding source allocation by a FIB-MST ratio approach.[34] Inconsistent ratios between FIB, MST markers, and pathogens in terms of both loads and persistence have been noted in other studies with feces and environmental matrices.[27,28,35,36] QMRA models offer a path forward by utilizing distributions and probabilities rather than fixed values. Such models can include a blend of fecal and nonfecal sources of FIB, including eelgrass,[6] which is attractive given the growing concern over sand and vegetation as exposure routes.[5,37,38]

QMRAs based on MST marker measurements and literature pathogen values have been used to interpret water quality exceedances in terms of health risk,[39,40] providing a cost-effective alternative to direct pathogen measurements. At ICB, HumMST values ranged from <15—255 copies/100 mL (Table S5). Surprisingly, the highest HumMST concentration was measured at the open-wave beach (station SDS7, 2036 copies/100 mL, Table S5). A formal QMRA has yet to be performed at this site; however, these concentrations are below the reported 4,200 copies/100 mL associated with a benchmark illness rate of 30 GI illnesses per 1000 swimmers (~35 CFU ENT/100 mL) based on QMRA analysis of human marker in raw sewage.[39]

MST-based QMRA is an attractive first step here because most of the gull-associated pathogen measurements fell either above or below the detection limit (Table S10). Measured gull marker concentrations (Table S5) were similar to median concentrations reported for seawater and wet sand at another

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California beach.[30] Gull marker concentrations in kelp measured in that study (5×10^3 copies/dry)[30] were lower than median gull marker concentrations in eelgrass measured here (2×10^4 and 9×10^4 copies/dry g for fresh and wrack, respectively). Gull marker in excess of 4×10^6 copies/100 mL provided a median predicted illness rate greater than 30 illness/1000 swimmers, but that QMRA analysis[7] used a different MST assay (CAT[41] versus Gull2Taqman, Table S3). In gull feces, the median marker concentration measured in this study (5×10^6 copies GullMST/g wet, Table S9) was similar to CAT measured in gull feces in another study (1×10^6 copies CAT/g wet[41]) but was less than CAT in gull feces collected for the QMRA analysis[7] (2×10^8 copies CAT/g wet). Both markers target similar portions of the *C. marimammalium* genome,[23] and similar performance metrics have been reported.[42] Conducting a QMRA based on GullMST marker and comparison to CAT marker is an area requiring future research.

Despite improvement in water quality after management action to exclude gulls, the zero exceedance goals of the TMDL were not met (Figure 4). Failure to meet objectives could be due to the structure being static; gulls have been observed to acclimate even to falcons unless the falconry was active.[10] It is also possible that reaching a goal of zero exceedance is not feasible. Although the TMDL uses a reference beach concept grounded in an antidegradation approach;[43] that approach was originally designed to discourage point source discharge into high quality waters[44] "where existing quality is higher than necessary for the protection of beneficial uses".[45]

The NSE provision includes reconsidering TMDL stipulations in case the reference beach/antidegradation approach is not suitable.[8,9] The TMDL for this semienclosed beach mandates that the enterococci geometric mean never exceed 35 MPN/100 mL, and no single sample may exceed 104 MPN/100 mL during summer dry conditions.[13] However, even open-wave reference beaches exceed the single sample maximum for ENT during drought conditions (typically 0% but as high as 40% per month at open-wave beaches; 0−100% in a creek-surf mixing zone; 80−100% in an estuary), and detections of human MST marker of up to 10% have been reported.[29] This information is expected to be used in future evaluations of the TMDL stipulations.[9] In addition, the TMDL criteria[13] are stricter than federal guidance, which offers the enterococci single sample criteria as the 75th percentile of the distribution that provided the enterococci geomean of 35 CFU/100 mL;[46] therefore, 25% of samples would be expected to exceed 104 MPN/100 mL, with zero exceedance expected for a geometric mean <2 CFU/100 mL.[47] Newer federal guidelines offer that 10% of samples may exceed 130 CFU/100 mL.[48]

FIB exceedances are not easily ignored and human marker detection can cause concern even at low concentrations, despite potential overprotection in stipulations. This presents a conundrum for managers tasked with balancing the demands of public safety and wise use of public funds. MST can identify contamination sources and inform remediation strategies to improve water quality and coastal economies. QMRA can help translate concentrations of FIB and MST markers into human health risk, although further research is needed to ensure that uncertainty can be bound adequately for QMRA to inform management.[49] If successful, MST-informed QMRA is expected to be increasingly used to devise, implement, and reconsider bacteria TMDLs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b05886.

Figure S1, Overview of the study area; Figure S2, Boxplots of FIB, ENTqPCR, and MST Marker, cross-shore transect; Figure S3, ICB geomean concentrations across survey; Table S1, survey dates and conditions; Table S2, sampling station locations and descriptions; Table S3, real-time PCR assays and parameters; Table S4, range and standard curve metrics for qPCR analyses; Table S5, results by matrix, site, and parameter; Table S6, results for compliance samples collected during FIB exceedance; Table S7, FIB and MST correlations for ICB seawater and wet sand; Table S8, pathogen and FIB concentrations in seawater by culture analysis; Table S9, pathogen and FIB concentrations in gull feces by culture analysis; Table S10, MST markers and enterococci in gull feces by qPCR; Table S11, pathogen, FIB, and MST in cat feces; Table S1, pathogen, FIB, and MST results for raccoon feces; Table S13, summary of concentrations of pathogens and FIB in seawater and feces; and Table S14, sanitary sewer assessment results for groundwater and swash zone stations (PDF)

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