United States Department of the Interior

U. S. GEOLOGICAL SURVEY
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January 11, 2010

Mr. James Thorsen,
City Manager,
City of Malibu,
23815 Stuart Ranch Road,
Malibu, California 90265

Dear Mr. Thorsen;

Attached are the results of human-specific *Bacteroidales* and *Enterococcus* qPCR data collected by the U.S. Geological Survey (USGS) and analyzed by the University of California Santa Barbara (UCSB) as part of our cooperative work in the Malibu area. Human-specific *Bacteroidales* are a measure of fecal contamination from human sources. The UCSB report (Attachment A) describes sample processing and results. Results for human-specific *Bacteroidales* data also are given in Table 1 (modified from Table 3 in the UCSB report). Sample site locations are shown in figures 1 and 2.

Human-specific *Bacteroidales* only were quantifiable in samples collected within the two septic systems sampled in Malibu Colony (MC-OLD-Septic and MC-ADV-Septic). Human-specific *Bacteroidales* concentrations were higher in the sample collected from within the traditional septic system (MC-OLD-Septic) than the sample collected within the advanced septic system (MC-ADV-Septic). High concentrations of human-specific *Bacteroidales* in samples from septic system are not unexpected. Human-specific *Bactertoidales* were present but not quantifiable from one well (P-9) near commercial septic discharges adjacent to Malibu Lagoon, and in water extracts from kelp and sand (Kelp extract and Sand extract). Human-specific *Bacteroidales* were not detected in other groundwater samples, samples from Malibu Lagoon, and samples from near-shore ocean water.

If you have any questions, please contact me at (619) 225-6131.

Sincerely

John Izbicki
Research Hydrologist

Table 1. Human-specific *Bacteroidales* (HBM) concentrations near Malibu, California, July 2009

[ND = not detected. DNQ = detected but not quantifiable.]

Site identification (figs. 1 and 2)	Date	Time	qPCR Dilution (1:x)	HBM Copies per liter	Standard error
Samples from wells and piezometers					
P-9	7/22/2009	10:00	5	DNQ	-
C-1	7/26/2009	11:45	5	ND	-
SMBRP-13	7/22/2009	14:30	10	ND	-
SMBRP-12	7/22/2009	10:30	5	ND	-
SMBRP-2	7/23/2009	13:15	5	ND	-
ML-BERM-Pz5'	7/23/2009	21:00	5	ND	-
Seepage-Deep	7/24/2009	6:00	5	ND	-
MC-ADV-Pz	7/25/2009	6:00	5	ND	-
MC-OLD-Pz	7/25/2009	6:00	10	ND	-
Samples from the near-shore ocean					
ML-BERM-OF (low tide)	7/24/2009	6:00	5	ND	-
MC-ADV-OF (low tide)	7/25/2009	6:00	10	ND	-
MC-OLD-OF (low tide)	7/25/2009	6:00	10	ND	-
MC-OLD-OF (high tide)	7/25/2009	13:00	10	ND	-
Samples from Malibu Lagoon					
ML-BERM	7/23/2009	21:00	10	ND	-
ML-Comm	7/24/2009	11:20	10	ND	-
ML-W	7/26/2009	12:45	5	ND	-
Samples from septic systems and special sources					
MC-OLD-Septic	10/1/2009	12:30	10	7.6E+07	1.3E+06
MC-ADV-Septic	10/1/2009	11:00	10/5*	4.2E+04	3.7E+03
Kelp extract	7/24/2009	17:00	10	DNQ	-
Sand extract	10/1/2009	8:00	5	DNQ	-

*Malibu Adv Septic, when run at 10 fold dilution was not within quantifiable range of the HBM qPCR assay. When run at 5 fold dilution, the sample results were within quantifiable range despite inhibition in this dilution in salmon testes qPCR.

DNQ—Detected but not quantifiable. Human-specific *Bacteroidales* is present in the sample but the concentration was less than the quantification limit obtainable from the laboratory standards. The DNQ concentration is dependent on sample volume, the amount of DNA extracted, and the dilution required to eliminate sample inhibition during the PCR reaction. The DNQ varied from sample to sample but the quantification limit would commonly be about 10^3 copies per liter.

ND—Not detected. Human-specific *Bacteroidales* was not detected in the sample. The ND concentration is dependent on sample volume, the amount of DNA extracted, and the dilution required to eliminate sample inhibition. The ND varied from sample to sample. Assuming 1 copy of *Bacteroidales* DNA per sample tray, a 5 to 1 dilution to eliminate inhibition during the PCR reaction, the addition of 2.5 µl of reagents, a 1-L sample containing 50 µg of DNA, and 100 percent efficiency in the PCR reaction—the limit of detection would be about 25 copies per liter. For a 10 to 1 dilution, the limit of detection would be about 50 copies per liter.

Figure 1.—Location of groundwater sampling sites, Malibu California, July 2009

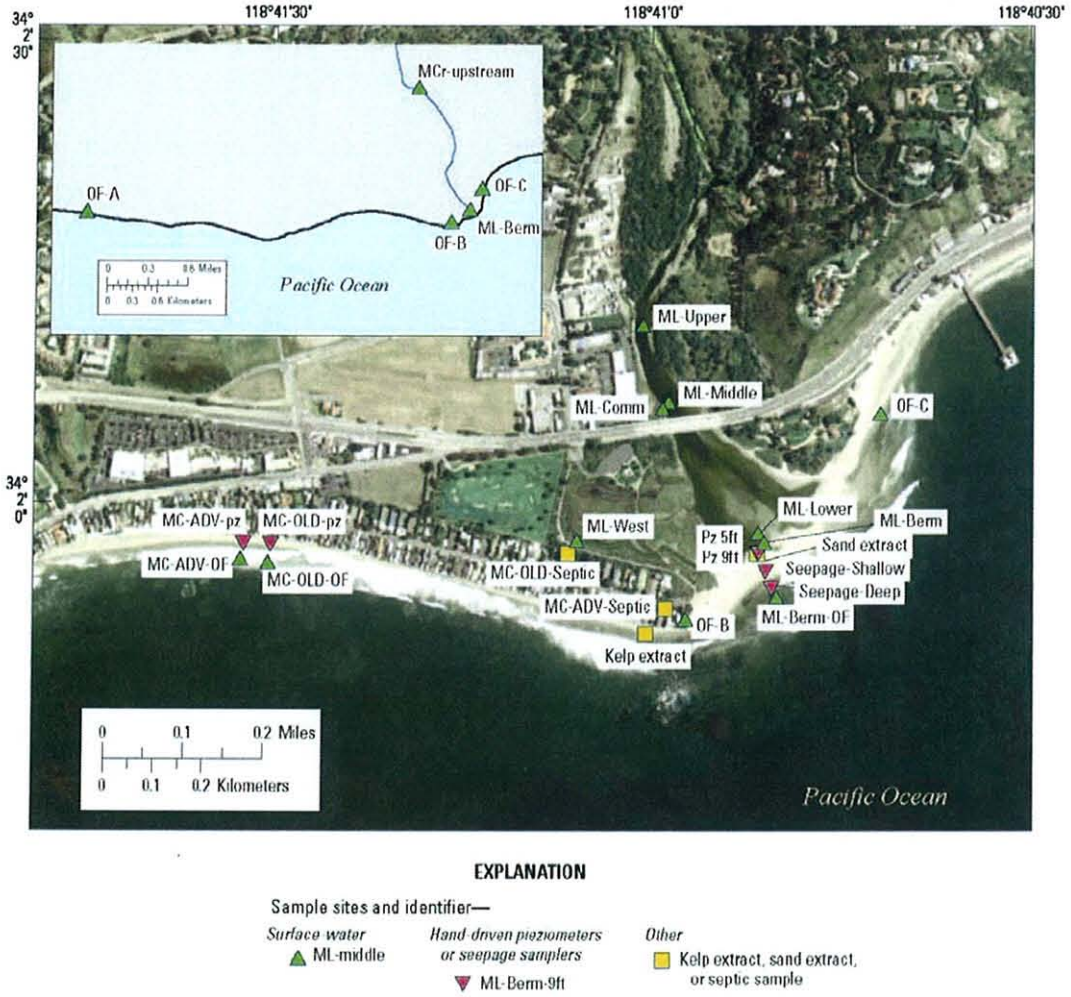

Figure 2.—Location of surface-water sampling sites, Malibu California, July 2009

Attachment A

University of California data transmittal

Human-specific *Bacteroidales* and *Enterococcus* qPCR results for USGS Malibu samples (7/22/09 – 10/1/09)

Report to John Izbicki USGS
December 11, 2009

Performed in the Laboratory of Patricia Holden, Donald Bren School of Environmental Science & Management, UC Santa Barbara (holden@bren.ucsb.edu, 805-893-3195) by Laurie Van De Werfhorst, Staff Research Associate II.

1. Sample processing

Twenty samples were received between 7/22/09 and 10/1/09 (Table 1). Upon arrival at UCSB, each sample was immediately filtered through 0.22 µm filters included in the extraction kit (PowerWater® DNA Isolation Kit, MoBio Laboratories, Inc.). Filtration was carried out until the entire sample was filtered or reached the point of refusal. Actual volume filtered was recorded, and the filters were removed using sterile forceps and stored at -20°C in the bead beating tubes provided with the extraction kit.

Table 1. USGS sample ID, sampling date and time, and actual volume filtered for each of the 20 samples received between 7/22/09 and 10/1/09.

USGS ID	Date	Time	vol.filtered L
P-9	7/22/2009	10:00	1.860
SMBRP-13	7/22/2009	14:30	1.880
SMBRP-12	7/22/2009	10:30	1.870
SMBRP-2	7/22/2009	13:15	1.900
ML-BERM	7/23/2009	21:00	0.360
ML-BERM-Pz5'	7/23/2009	21:00	1.880
ML-BERM-OF	7/24/2009	6:00	1.440
ML	7/24/2009	11:20	0.570
ML-BERM-PzD	7/24/2009	6:00	1.890
Kelp extract	7/24/2009	17:00	0.065
MC-ADV-OF	7/25/2009	6:00	0.990
MC-HT	7/25/2009	13:00	1.160
MC-ADV-Pz	7/25/2009	6:00	1.920
MC-OLD-Pz	7/25/2009	6:00	1.850
MC-OLD-OF	7/25/2009	6:00	1.030
C-1	7/26/2009	11:45	1.865
ML-W	7/26/2009	12:45	0.640
MC-OLD-Septic	10/1/2009	12:30	0.120
MC-ADV-Septic	10/1/2009	11:00	0.100
Sand extract	10/1/2009	8:00	0.960

2. DNA Extraction

The PowerWater® DNA Isolation Kit (MoBio Laboratories, Inc.) was used to extract DNA from the frozen filters. DNA was extracted following the manufacturer's protocol, followed by ethanol precipitation. Total DNA was quantified using the Quant-iT™ dsDNA BR Assay Kit (Invitrogen) and related to the volume of water filtered for reporting DNA concentration (DNA yield).

3. qPCR

All TaqMan qPCR analyses were performed in a CFX96 BioRad thermocycler, using dual-labeled (BHQ-FAM) probes (Eurofins MWG Operon). The qPCR MasterMix Plus (no ROX) (Eurogentec) was used in final reaction volumes of 25 µl, including 2.5 µl of diluted DNA template. The thermocycling program for all analyses was: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 sec at 94 °C and 60 sec at 60 °C.

Salmon testes qPCR

The Taqman qPCR assay for salmon testes DNA was performed prior to *Enterococcus* spp. and human-specific *Bacteroidales* qPCR assays (ENT qPCR and HBM qPCR respectively) in order to determine the lowest template dilution without inhibition. This assay was based on the protocols by (Haugland et al., 2005) and (Morrison et al., 2008). The qPCR master mix was spiked with salmon testes DNA, to a final concentration of 0.25 ng/reaction. Four no-sample DNA reactions (= no inhibition control) are run on each plate, in which only salmon testes DNA, PCR reagents and PCR-grade water are added. In addition, a 3 log salmon testes DNA standard curve was run to determine amplification efficiency. Diluted template DNA (2.5 µl) was added to all remaining reactions (in duplicate). Using the no-inhibition controls, the average + 3 × standard deviation cycle threshold value (Ct_{ni}) was calculated. This value was used as the upper Ct value for no inhibition. All reactions with sample DNA that produced an average $Ct > Ct_{ni}$ were considered to be inhibited. The assay was first run using 1:5 diluted DNA template to determine the occurrence of reaction inhibition. If inhibition occurred, twofold dilutions were analyzed until no inhibition occurred. The lowest template dilution without inhibition was used for ENT and HBM qPCR assays.

HBM qPCR

The assay for human-specific *Bacteroidales* qPCR was adopted from the published literature (Kildare et al., 2007) using TaqMan chemistry and qPCR parameters as detailed above. The primer and probe concentrations used were 400 nM (primers) and 80 nM (probe). A human-specific *Bacteroidales* marker (HBM) standard was previously created by purifying PCR products from amplified DNA extracted from multiple sewage samples. Standard concentrations ranged from 1E+06 to 1E+01 markers/reaction. All samples and standards were run in triplicate. Each plate was standardized by adjusting the baseline threshold position until the Ct values for the standard dilutions were less than 3% from run to run. The resulting sample Ct values were then used to calculate the number of HBM per liter of sample filtered, and the triplicate values for each sample were averaged. Samples with 2 or more replicates that amplified after the lowest standard were considered "detected but not quantifiable" (DNQ).

ENT qPCR

The *Enterococcus* spp. qPCR assay is based on a published protocol (Haugland et al., 2005) using TaqMan chemistry and qPCR parameters as detailed above. The primer and probe concentrations used are 900 nM (forward primer), 300 nM (reverse primer) and 100 nM (probe). Standard concentrations ranged from 1E+06 to 2.5E+01 markers/reaction. All samples and standards were run in triplicate. Each plate was standardized by adjusting the baseline threshold position until the Ct values for the standard dilutions were less than 3% from run to run. The resulting sample Ct values were then used to calculate the number of *Enterococcus* spp. (ENT) markers per liter of sample filtered, and the triplicate values for each sample were averaged. Samples with 2 or more replicates that amplified after the lowest standard were considered "detected but not quantifiable" (DNQ). The cell equivalents (c.eq.) per 100 mL were also calculated, by assuming an *rrn* operon copy number of 6 for *Enterococcus*.

4. Results

DNA yield

The calculated DNA yield ranged from 134 ng/L filtered (P-9) to 122,308 ng/L (Kelp-1) (Table 2).

Table 2. DNA yield (ng DNA per liter sample filtered).

USGS ID	DNA yield ng/L filtered
P-9	134
SMBRP-13	559
SMBRP-12	294
SMBRP-2	211
ML-BERM	13889
ML-BERM-Pz5'	559
ML-BERM-OF	972
ML	7544
ML-BERM-PzD	450
Kelp extract	122308
MC-ADV-OF	2778
MC-HT	3534
MC-ADV-Pz	781
MC-OLD-Pz	1622
MC-OLD-OF	3738
C-1	1046
ML-W	6484
MC-OLD-Septic	49167
MC-ADV-Septic	24000
Malbu extract	781

HBM qPCR

Approximately one half of the samples showed no inhibition in the salmon testes qPCR when run at a 1:5 dilution. The remaining samples needed to be run at a 1:10 dilution in order to remove inhibition. HBM qPCR was run with the optimal sample dilution as determined by salmon testes qPCR, on a sample by sample basis (Table 3).

Table 3. Optimal dilution for qPCR as determined by salmon testes qPCR, and human-specific *Bacteroidales* (HBM) concentrations (copies/L and standard error (SE)). ND = not detected. DNQ = detected but not quantifiable.

	qPCR Dilution	HBM	
USGS ID	(1:x)	Copies/L	SE
P-9	5	DNQ	-
SMBRP-13	10	ND	-
SMBRP-12	5	ND	-
SMBRP-2	5	ND	-
ML-BERM	10	ND	-
ML-BERM-Pz5'	5	ND	-
ML-BERM-OF	5	ND	-
ML	10	ND	-
ML-BERM-PzD	5	ND	-
Kelp extract	10	DNQ	-
MC-ADV-OF	10	ND	-
MC-HT	10	ND	-
MC-ADV-Pz	5	ND	-
MC-OLD-Pz	10	ND	-
MC-OLD-OF	10	ND	-
C-1	5	ND	-
ML-W	5	ND	-
MC-OLD-Septic	10	7.6E+07	1.3E+06
MC-ADV-Septic	10/5*	4.2E+04	3.7E+03
Malbu extract	5	DNQ	-

*Malibu Adv Septic, when run at 10 fold dilution was not within quantifiable range of the HBM qPCR assay. When run at 5 fold dilution, the sample results were within quantifiable range despite inhibition in this dilution in salmon testes qPCR.

Two of the 20 samples analyzed had HBM within quantifiable range of the assay: MC-OLD-Septic and Malibu Adv Septic. The Malibu Adv Septic sample, when assayed at the optimal dilution of 10, had 2 of 3 replicates amplify just past the last standard and the third replicate with the last standard. Since the resulting HBM qPCR product was so close to the quantification limit, the sample was reanalyzed at 5 fold dilution and was able to be quantified despite the inhibition present in the 1:5 dilution in the salmon testes qPCR.

For a few other samples (P-9, Kelp-1, Malibu Sand), there was also amplification in 2 or more replicates past the limit of quantification (~10 copies/well) (Table 3). However, the amplification amount was well below the lowest standard, even more so when compared to the Malibu Adv Septic sample. Thus, for these samples, it was judged that adding more

template DNA or amplifying undiluted sample would not necessarily bring the samples within a quantification range.

Samples reported without the HBM in a quantifiable range do not necessarily indicate a lack of fecal contamination. It is possible that dilution to remove inhibition may have reduced the sensitivity of the assay by diluting the number of HBM in the sample below the quantification limit.

ENT qPCR

The same sample dilutions used in the HBM qPCR assay (as determined by the salmon testes qPCR assay) were used in the ENT qPCR assay. Ten of the 20 samples analyzed had ENT concentrations within the quantifiable range of the assay (Table 4).

Table 4. Salmon testes qPCR determined optimal dilution (same as in Table 3), and *Enterococcus* spp. (ENT) qPCR concentrations (copies/L with standard error (SE), and cell equivalents (c.eq.)/100 mL with SE). ND = not detected. DNQ = detected but not quantifiable. Calculation of c.eq. per 100 mL assumes 6 copies of 16S rDNA per cell.

	qPCR Dilution	ENT			
USGS ID	(1:x)	Copies/L	SE	c.eq./100 mL	SE
P-9	5	DNQ	-	DNQ	-
SMBRP-13	10	DNQ	-	DNQ	-
SMBRP-12	5	DNQ	-	DNQ	-
SMBRP-2	5	DNQ	-	DNQ	-
ML-BERM	10	4.3E+04	4.0E+03	719	66
ML-BERM-Pz5'	5	1.5E+03	5.3E+01	24	1
ML-BERM-OF	5	3.2E+03	2.2E+02	54	4
ML	10	1.3E+04	1.1E+03	221	18
ML-BERM-PzD	5	DNQ	-	DNQ	-
Kelp extract	10	1.3E+06	5.6E+04	21289	927
MC-ADV-OF	10	DNQ	-	DNQ	-
MC-HT	10	DNQ	-	DNQ	-
MC-ADV-Pz	5	DNQ	-	DNQ	-
MC-OLD-Pz	10	6.3E+04	3.9E+03	1049	65
MC-OLD-OF	10	DNQ	-	DNQ	-
C-1	5	2.0E+03	3.2E+02	34	5
ML-W	5	2.7E+04	1.6E+03	456	27
MC-OLD-Septic	10	5.2E+07	1.7E+07	868963	276148
MC-ADV-Septic	10/5*	DNQ	-	DNQ	-
Malbu extract	5	1.7E+05	6.6E+03	2823	110

*Malibu Adv Septic, when run at 10 fold dilution was not within quantifiable range of the ENT qPCR assay. When run at 5 fold dilution, the sample results were still not in quantifiable range.

The Malibu Adv Septic sample, when assayed at the optimal dilution of 10, amplified after the lowest standard. Even though this amplification was not as close to the lowest standard as it was for this sample in the HBM assay, the Malibu Adv Septic sample was

reanalyzed at 5 fold dilution in the ENT assay as well. However, the lesser dilution was still unable to bring the sample within quantifiable range.

For the other 10 samples, there was amplification in 2 or more replicates past the limit of quantification (~25 copies/well) (Table 4). However, the amplification amount was well below the lowest standard and the Malibu Adv Septic sample. Therefore it was judged that for these samples, adding more template DNA or amplifying undiluted sample would probably not bring them within a quantification range.

5. References

Haugland, R. A., S. C. Siefring, et al. (2005). "Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis." Water Research **39**: 559-568.

Kildare, B. J., C. M. Leutenegger, et al. (2007). "16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: A Bayesian approach." Water Research **41**(16): 3701-3715.

Morrison, C. R., D. S. Bachoon, et al. (2008). "Quantification of enterococci and bifidobacteria in Georgia estuaries using conventional and molecular methods." Water Research **42**(14): 4001-4009.