On-Line Supplemental Information for

Impacts of Hurricanes Katrina and Rita on the Microbial Landscape of the New Orleans Area

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Materials and Methods

Remote Sensing Image Analysis for Inundation Depths and Flood Volume Computations

Computations: The flooding in New Orleans is clearly delineated on a pan-sharpened color-infrared SPOT satellite image taken 30 August 2005. A digital map of flood depth on the NE bank of the Mississippi River was constructed by combining observed areas of flooding on the SPOT image with high resolution digital elevation data (*1*). Elevations were extracted along both the edge of flooding and on exposed highway surfaces in the flooded interior to create a set of flood heights. These heights varied spatially throughout the study area, and were used to construct a model of flood height composed of several contiguous polygons of constant height. The boundaries between polygons generally corresponded to barriers to water flow such as levies, floodwalls, highways, or natural topographic ridges. Finally, a raster model of flood depth was calculated by subtracting a 10 m resolution digital elevation model extracted from the USGS National Elevation Database (http://seamless.usgs.gov) from the flood height model.

Sample Collection: Surface water samples from Lake Pontchartrain and from canals were collected in sterile 10L polypropylene carboys, polypropylene bottles, or acid-washed and ethanol-rinsed polyethylene cubitainers® (Hedwin Corporation) from the locations indicated in Fig. 1 and Table S-1. Water samples were kept in a cooler with ice packs maintained between 12 and 24 °C (*Vibrio* analyses) or on ice (all other analyses) until return to the laboratory within approximately 4-6 hours. Approximately 100g sediment samples, both from canal shorelines and from dried floodwater sediment deposits in residential yards, were collected into sterile 250mL specimen cups from the top 3-5 cm of sediment using sterile plastic spatulas. In the case of shoreline samples from lakes and canals, the sediment was collected from just above the water line.

Sediment samples were also kept on ice until return to the laboratory. Additional environmental sampling was also conducted at this same time that will be reported elsewhere. Upon return to the laboratory, water samples were used directly for processing and analysis by chromogenic substrate assay (IDEXX, Westbrook, ME), plate count enumeration, and extraction of nucleic acids. In the case of sediment samples, 30g of sediment were resuspended in 300mL of sterile 1X PBS solution in sterile Whirlpak® bags (Nasco, Ft. Atkinson, WI), then sealed and vigorously agitated and kneaded to resuspend the bacteria into the PBS. The resuspended samples were allowed to settle for 10 minutes, then the supernatant was processed for chromogenic substrate, and nucleic acid extraction in a similar fashion to the environmental water samples.

Total Microbial Community DNA Extractions: Two different collection and DNA extraction methods were employed: (1) Filtration through polyethersulfone membrane capsule filters (0.22 µm pore size Sterivex™, Millipore, Billerica, MA) followed by DNA extraction and purification using a modified salt precipitation method (PUREGENE®, Gentra Systems, Minneapolis, MN). This high-yield method was used for community sequence analysis, for pathogen screening, and DNA archiving. (2) Filtration through flat polycarbonate (PC) track-etched membranes (0.45 µm pore size, 47 mm diameter; GE Osmonics, Minnetonka, MN) followed by cell lysis using a bead-based homogenizer (FastPrep®, Qbiogene, Solon, OH) and DNA purification using a silica-based procedure (FastDNA® Spin Kit for Soil, Qbiogene). This method was used for samples to be analyzed by quantitative real-time PCR (qPCR).

For the first method, subsamples (1 L) of lake and canal water were filtered through the Sterivex™ filter capsules using positive pressure with a peristaltic pump or by applying vacuum to the filter outlet. Filters were pumped to dryness then filled with

2mL of lysis buffer from the PUREGENE® Genomic DNA Isolation Kit, sealed with Luer end caps, wrapped in parafilm, and then stored frozen at -80ºC until further processing. Filter capsules were subsequently thawed and 10µL of Lytic Enzyme solution (supplied with kit) was added. Filters were resealed and incubated at 37ºC for 30 min on the rotating carousel of a hybridization oven. Ten microliters of Proteinase K solution (supplied with kit) was then added to the filters, which were resealed and incubated at 65ºC for 1 h with rotation in a hybridization oven. The lysate from all the filters for a given sample was withdrawn using a 3cc syringe and combined into a 15 ml tube, cooled to room temperature and then DNA was purified from each pooled sample according to PUREGENE® kit manufacturer's instructions. Resulting DNA was quantified and archived at -80ºC for subsequent analysis.

For the second method, bacteria from 100mL water samples were collected on flat PC filters, which were then transferred to the vials of "lysing matrix E" from the Fast DNA Spin Kit for Soil (QBiogene, Irvine, CA), and one *Lactococcus* Sample Prep Control SmartBead™ (Cepheid) containing lyophilized *Lactococcus* control cells at a known concentration was added to each vial. The DNA from the PC filters (along with DNA from the *Lactococcus* SPC bead) was then extracted in these bead-beating vials using the Fast DNA Spin Kit for Soil (QBiogene) according to manufacturer's instructions, except that the filter replaced the sediment pellet. Eluted DNA was stored at -80ºC for subsequent analysis by qPCR. In the case of sediment samples, 100mL of the supernatant of resuspended sediment bacteria in 1X PBS was collected on PC filters and extracted with the Fast DNA Spin Kit for Soil as described above for water samples.

Enterococci and *E. coli* Using Membrane Filtration Method: Standardized methods were utilized (Method 1600 (*2*) and Method 1103.1 (*3*)) with the processing of water

samples within 6 hours. In brief, the method requires the filtration of the water sample through a membrane which captures the bacteria. This membrane is then placed on the selective medium. For enterococci, mEI media (membrane-Enterococcus Indoxyl-b-D-Gluocoside) and for *E. coli*, mTEC media (membrane-Thermotolerant *Escherichia coli*) were used. The enterococci plates were incubated at 41^{o}C for 24h, and colonies with blue halos counted. The *E. coli* plates were incubated at 35^{o}C for 2h and then incubated at 44.5^{o}C for 22h. After incubation, the filter was transferred to a pad saturated with urea substrate and yellow, yellow-green and yellow brown colonies were counted after 15 min. Both *E. coli* and enterococci were reported as CFU/100ml.

Enterococci Using Chromogenic Substrate Method: Duplicate 10-fold, 100-fold, and 1000-fold dilutions of each water sample were prepared in sterile 1X PBS. Each dilution was then processed and assayed using the Enterolert™ enterococci test kit and Quanti-Tray/2000 (IDEXX, Westbrook, ME), according to manufacturer's directions. In the case of sediment samples, duplicate dilutions in sterile 1X PBS were made of the initial sediment bacterial resuspension described above, processed, and assayed in the same manner as water samples with the chromogenic substrate method.

Enterococci and *E. coli* Using Quantitative PCR Method: Ten-fold and 100-fold dilutions of DNA extracts from water or sediment samples were analyzed for relative abundance of enterococci and *E. coli* using real-time quantitative PCR water quality assay kits from Cepheid (Sunnyvale, CA).

For enterococci enumeration, 5µL samples of each DNA dilution from the PC filter extracts was amplified using the "Total *Enterococcus* Scorpion® Primer and Probe Set and Positive Control DNA" beads and the "OmniMix-HS™ Lyophilized PCR Master

Mix" beads according to manufacturer's instructions. Each Total *Enterococcus* assay bead contains a proprietary mix of fluorescently labeled Scorpion primers targeting *Enterococcus*-specific portions of the 16s rDNA, internal amplification control DNA, and a second set of Scorpion primers that target the internal control DNA all lyophilized together in amounts sufficient for four 25µL reactions per bead. The Omnimix-HS™ beads (Cepheid) contain all other buffers, reagents, nucleotides, enzymes, (except for water and target DNA) lyophilized into a bead for two 25µL reactions. Amplifications were done in batches of 16 reactions on a SmartCycler™ real-time PCR thermocycler (Cepheid), by dissolving 4 enterococci primer beads with 8 OmniMix-HS™ beads, and 340µL of sterile molecular-grade water. This mixture was aliquotted in 20µL volumes into sterile Smartcycler-Tubes™ (Cepheid), 5µL of template DNA was added to each tube, which was then sealed, centrifuged, and cycled using the following parameters: 45 cycles of 95ºC for 5 sec and 62ºC for 43 sec, with the FCTC25 dye set calibration, FAM threshold manually set at 7, and other settings at default. For each amplification set, negative controls of sterile water in place of DNA template were run, as well as positive controls of known amounts of DNA from *Enterococcus faecalis*. Standard curves for quantitation were run in duplicate and were generated by using 5 serial dilutions of "Enterococcus Postive Control DNA Beads" (Cepheid) which consisted of lyophilized beads containing *E. faecalis* DNA of known concentration that had been calibrated to CFU equivalents of enterococci. Quantitation of enterococci in unknown samples could thus be determined using these standard qPCR amplification curves.

In a similar fashion, *E. coli* abundance was enumerated using dilutions of template DNA from the PC filter extracts to be amplified by qPCR using the conditions described above for *Enterococcus*, but using "*E. coli* Species Scorpion Primer and Probe Set with Positive Control DNA" beads (Cepheid), and standard curves generated from

"*E. coli* Postive Control DNA" beads (Cepheid). Assay and cycling conditions were the same as for the *Enterococcus*.

For the *Lactococcus* Sample Processing Control (SPC), dilutions of template DNA from the PC filter extracts were amplified in a similar manner as above, except that the primers beads used were from the "*Lactococcus* SPC SmartBeads: Scorpion Primer and Probe Set with Positive Internal Control DNA."

Clostridium perfringens: Water samples for *C. perfringens* were assayed using membrane filtration on mCP agar (*4*). Sample aliquots of 100 ml were placed into a water bath and pasteurized for 15 minutes at 60^{o}C. 25 ml aliquots of pasteurized samples were filtered thru 0.45 µm pore size membranes (GN-6 Metricel; Pall Gelman, East Hills NY) and placed filter side up onto mCP agar. Agar plates were incubated anaerobically at 45^{o}C for 24 hours. After 24 hours, plates containing yellow (sucrose positive) colonies were exposed to ammonium hydroxide fumes for 20 seconds and resulting pink or magenta colonies were counted.

FRNA coliphages: FRNA coliphages were assayed on *E. coli* HS(pFamp)R using the most probable number assay described by Sobsey *et al.*, (*5*). The assay involves enrichment of 100 mL of water which are subsequently divided into aliquots of 30 (3), 3 (3) and 0.3 (3) mL. These aliquots are seeded with *E. coli* HS(pFamp)R (0.5 mL) and enriched with 10X concentrated TSB (5 mL) with Ampicilin and Streptomicin (100X) including addition of $MgCl_2$ (4 M) (1.25 mL). Aliquots were incubated for 24 h at 37 ºC. After the incubation period, subsamples are spun at high speed and aliquots of 5µL were spotted onto a lawn of *E. coli* HS(pFamp)R. Lysis zones were recorded and positive or

negative results were entered into a MPN program to derive a most probable number of plaque-forming units (PFU) along with a 95% confidence interval (*5*).

Bacteroidales: Fecal *Bacteroidales* as a measurement of general fecal pollution was measured by standard PCR using reamplification or by real time PCR using the *Taq* nuclease assay (TNA) described by Dick and Field (*6*) which targets a segment of the *Bacteroidales* 16S rRNA gene. The combination of primers and fluorogenic probe enables amplification of 16S rDNA of *Bacteroidales* from human, cow, dog, cat, pig, elk, deer and gull feces. Although the TNA was originally developed as a quantitative assay, the data derived from water samples reported here were based on presence/absence evaluations.

***Bifidobacterium*:** A nested PCR protocol to detect *B. adolescentis* was performed on the extracted DNA samples as described by King, et al. (*7*). The specificity of this assay has previously been tested against feces from human, cow, horse, pig, dog, chicken, and goose, as well as against sewage samples and environmental water samples known to be contaminated with either human or animal feces, and has only shown detection of human-source fecal contamination.

The first step consisted of an amplification using the universal 16S rRNA primer, 785R (CTACCAGGGTATCTAATCC) and the Bifidobacterium genus specific primer, IM26F (GATTCTGGCTCAGGATGAACG). Each PCR reaction contained a 30µL volume with 0.3 mM dNTP, 3 mM $MgCl_2$, 1 U Taq DNA polymerase, 1X PCR reaction buffer, and ~30 ng of template DNA. The samples were run on a thermocycler (Techne Model TC-312, Burlington, NJ) under the following conditions: initial denaturing at 94˚C for 5 min; 45 cycles of 94˚C for 30sec, 48˚C for 30sec, and 72˚C for 30sec; and

final elongation at 72˚C for 5 min. Product from this PCR reaction was then used as template for a second PCR mixture.

For this second PCR protocol, the template was amplified using *Bifidobacterium adolescentis* species-specific primers ADO1 (CTCCAGTTGGATGCATGT), and ADO2 (CGAAGGTTGCTCCCAGT) (*8*). One microliter of product from the first PCR was added to a 30µL reaction mixture containing the same concentrations of $MgCl_2$, 1X reaction buffer, dNTP, and Taq as above. These samples were run on the thermal cycler under the following conditions: initial denaturing at 94˚C for 5 min; 45 cycles of 94˚C for 30sec, 48˚C for 20sec, 55˚C for 30sec, and 72˚C for 1 min; and final elongation at 72˚C for 5 min. Products from both the first and seconds rounds of PCR were subjected to electrophoresis in a 1.5% agarose gel stained with ethidium bromide. The detection limit for this method was 200 *Bifidobacterium adolescentis* cells. This was determined using *B. adolescentis* genomic DNA ATCC ® number 15703D ™ .

***Vibrio* Species:** A portion of each water sample was diluted 1:100 into sterile solution of peptone (0.1% w:v) and NaCl (3% w:v) in water (PS; (*9*)). Twenty-five milliliters of undiluted or diluted sample were filtered in duplicate onto 0.45 µm pore size mixed cellulose ester membrane filters (GN-6 Metricel; Pall Gelman, East Hills NY). One of each of the duplicate filters was placed face up on Thiosulfate-Citrate-Bile salts-Sucrose (TCBS) agar (Accumedia Manufacturers, Inc. Lansing, MI) and the other on CHROMagar® Vibrio (DRG International Inc., Mountainside, NJ). Plates were incubated overnight at 37°C. Total colonies were enumerated and representative colonies from each medium were picked with sterile plastic loops and gridded onto fresh plates of the opposing medium (colonies from TCBS were gridded onto CHROMagar Vibrio and vice versa) and incubated overnight at 37°C. Initial putative species identifications were

based on color changes on both chromogenic media. Gridded colonies were picked with a sterile loop and resuspended in 1 ml of PS with glycerol (15% v:v) contained in sterile cryovials. The resuspended colonies were frozen on dry ice for shipment to Hawaii and then maintained at -80°C. Clonal populations were recovered from the glycerol stocks by serial streaking then maintained on T1N1 (10% NaCl, 10% Tryptone) agar slants overlaid with mineral oil.

For PCR assays, cells from clonal colonies were resuspended in 100µL TE buffer in thin-walled PCR tubes using sterile loops. Tubes were heated to 100°C for 10 min in a thermal cycler to release bacterial DNA then centrifuged to pellet debris. A portion of each supernatant was used as the template for subsequent PCR reactions. PCR-based assays were performed to determine presence or absence of intergenic transcribed spacer sequences diagnostic for *V. cholerae* (VcITS; (*10*)), a *V. parahaemolyticus*-specific thermolabile hemolysin gene (*tlh*)(*11*), or the *V. vulnificus*-specific hemolysin A gene (*vvh*A)(*12*). As a positive control, samples were amplified with general bacterial primers for the 16S rRNA gene (*13*) with minor modifications. Samples that failed to amplify with the 16S rRNA gene primers were cleaned using InstaGene™ Matrix (BioRad Laboratories, Hercules, CA) following the manufacturer's protocol for bacterial DNA isolation, except that the initial water wash steps were omitted. Instead, fifty microliters of cell suspension in TE buffer was added directly to 200 ul of InstaGene™ Matrix buffer. Samples were then heated for 15 minutes at 56°C, vortexed, then heated for 8 minutes at 100°C, vortexed, then centrifuged at 10,000 X g for 2 minutes. Twenty microliters of the cleaned sample was used in the PCR analysis.

PCR assays were performed with the Expand PCR System (Roche Diagnostics, Alameda, CA) and contained 1X reaction buffer, 1µL template DNA extract, 0.25 µM of each primer, and 1.5 mL $MgCl_2$. All PCR reactions were initiated with a 4 min hold at

95°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at a reactions-specific temperature for 1 min, and extension at 72°C for 1 min.

The primer sequences (all written 5'-3') and the annealing temperature used with each primer set are as follows: VcITS-F (TTA AGC STT TTC RCT GAG AAT G) and VcITS-R (AGT CAC TTA ACC ATA CAA CCC G) at 60°C annealing, TLH-F (AAA GCG GAT TAT GCA GAA GCA CTG) and TLH-R (GCT ACT TTC TAG CAT TTT CTC TGC) at 58°C, VVH-F (TTC CAA CTT CAA ACC GAA CTA TGA C) and VVH-R (ATT CCA GTC GAT GCG AAT ACG TTG) at 63°C annealing, and 27F-B (AGR GTT YGA TYM TGG CTC AG) and 1429R (GGY TAC CTT GTT ACG ACT T) at 50°C annealing.

Presence and size of amplification products was determined by agarose gel electrophoresis, staining with SYBRsafe fluorescent DNA stain (Invitrogen, Carlsbad, CA), and visualization and documentation using UV transillumination and digital image capture (VersaDoc 3000, BioRad Laboratories, Hercules, CA).

***Legionella*:** A nested PCR amplification procedure using primers designed for the genus *Legionella* (p1.2 & cp3.2 (*14*), LEG225 & LEG858 (*15*)) was used to detect the presence of *Legionella* species in surface water samples from the transect samples taken in Lake Pontchartrain. The primers used in this method are specific for members of the genus, and sequencing is necessary to determine whether the amplified fragments represent human pathogens. In addition, amplification using primers specific for a portion of the *mip* gene of *L. pneumophila* were carried out following the method of Templeton et al. (*16*). One microliter of DNA extract (diluted 1:100) was used as the template for the PCR reactions, and products recovered using the *L. pneumophila* specific primers were sequenced to confirm their origin and similarity to published *L. pneumophila* mip

sequences. Sequencing was carried out on PCR products recovered by band isolation (Zymoclean Gel DNA Recovery Kit; Zymo Research, Orange, CA) using the amplification primers and the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Reactions were run on an ABI377 and chromatograms were analyzed using Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI).

***Cryptosporidium* and *Giardia* species:** On December 17, 2006, water samples for the enumeration of *Cryptosporidium* oocysts and *Giardia* cysts were collected from both the outfall of the 17th St. Canal at Lake Pontchartrain, and from the 17th St. Canal at the up-stream (i.e. city) side of New Orleans Pump Station #6 (i.e. the Metaire Pump Station), while active pumping of water from the city interior was in progress. *Cryptosporidium* oocysts and *Giardia* cysts were harvested from the water and enumerated according to EPA Method 1623, using the Filta-Max™ filtration and elution system (IDEXX, Westbrook, ME). Filter processing, immuno-magnetic separation, and immuno-fluorescent antibody staining (IFA) with subsequent fluorescent microscopy analysis of oocysts and cysts by EPA Method 1623 (*17*) was conducted by BCS Laboratories, Inc (Miami, FL), a NELAC-certified laboratory. Spiked control samples for each sample location were prepared as per EPA Method 1623 with EasySeed™ *Cryptosporidium* and *Giardia* control (BTF Precise Microbiology, Inc., Pittsburgh, PA). Sample volumes filtered for the canal outfall site were 196.30 liters and 146.90 liters for the natural sample and spiked control sample, respectively. Sample volumes filtered for the 17th St. Canal at Pump Station #6 were 110.35 liters and 71.74 liters for the natural sample and spiked control, respectively.

Soil samples (200g each) from sites Y2 and Y3 were again collected on this date in the same manner as described in the "Sample Collection" section above. Enumeration of *Cryptosporidium* oocysts and *Giardia* cysts in soil samples was conducted commercially by BCS Laboratories, Inc. There is currently no EPA standard method for *Cryptosporidium* and *Giardia* analysis in soils. Oocysts and cysts were extracted from 25g sub-samples of soil using the NaCl flotation method described by Kuczynska and Shelton (*18*). Soil sub-samples (25g) were dispersed in 100mL of Tris-Tween 80 dispersing solution (50mM Tris and 0.5% [vol/vol] Tween 80) with magnetic stirring for 15 min, then sieved, washed, centrifuged, and finally the oocysts and cysts harvested by flotation in NaCl (S.G. = 1.21) as previously described (*18*). After final resuspension of oocyst/cyst pellets in 100uL of distilled water, the (oo)cysts were then stained by IFA and enumerated by fluorescent microscopy as described above for EPA Method 1623.

Microbial Assemblage Analysis: PCR amplifications employed universal primers targeting the V4 –V8 region of the SSU rRNA gene of eukaryotes, archaea and bacteria corresponding to *E. coli* reference positions 517 through 1391: 517F (5' GCCAGCAGCCGCGGTAA3') and 1391R (5'-GACGGGCGGTGTGTRCA-3'). PCR reactions were carried out using an Eppendorf Mastercycler under the following conditions: 35 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 2 min; 72°C for 10 min. An Invitrogen Purelink Purification kit (Invitrogen, Carlsbad, CA) was used to purify PCR products for cloning and exclude any fragments less than 300 base pairs. An A-tailing reaction and an additional purification with the Purelink kit preceded cloning using the Invitrogen TOPO TA kit.

A RevPrep Orbit robotic template preparation instrument served to isolate and purify plasmid DNA (Genomic Solutions, Ann Arbor, MI). Sequencing of plasmid DNA was carried out using an ABI 3730 (Applied Biosystems, Foster City, CA) sequencing machine. A total of 192 clones were sequenced from each library in the forward and reverse directions using M13F (5' GTAAAACGACGGCCAGT 3') and M13R (5'AACAGCTATGACCATG 3') primers. We subjected individual sequence reads to quality control, vector trimming and assembly using phred, crossmatch and phrap software (*19*,*20*,*21*). We then ran BLAST searches against the nucleotide and environmental sequence databases to identify the closest relatives of the sequences observed to those in GenBank. Copies of the BLAST report summaries are available on the Woods Hole Centers for Oceans and Human Health website (http://www.whoi.edu/science/cohh/whcohh/). Using the BLAST results, we separated out bacterial sequences from archaeal and eukaryotic sequences and further subjected these sequences to the Ribosomal Database Project (RDP) II's Classifier (*22*) to obtain phylogenetic information about our environmental sequence data. The final total numbers of bacterial clones varied between libraries. The data shown in pie charts (Fig. 4) represents an overview of the bacterial diversity with reference to the associated environments of the study sites from which top BLAST hits were obtained. We find this approach useful in making broad scale comparisons between clone libraries derived from different sites and different dates. The phylogenetic affinity of the potential pathogens or pathogen related sequences is derived from taxonomic information obtained from the RDP classifier using the default settings.

Chlorophyll-a Analysis: Water samples (200 ml) were filtered through GF/F glass fiber filters, which were then frozen at -80ºC until extracted. Filters were extracted in two

steps, first for 30 min with 10mL of dimethyl sulfoxide at 5ºC, and then overnight with an additional 15mL of 90% acetone at -20ºC. Extracts were measured fluorometrically before and after acidification for measurement of chlorophyll and phaeopigment concentration using a fluorometer (Turner Designs Model 10-000R, Sunnyvale, CA) equipped with an infrared-sensitive photomultiplier and calibrated using pure chlorophyll-*a*.

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