Newport Coast and Laguna Areas of Special Biological Significance Protection Program

Mussel Bioassay

Draft Final Report

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Background

The cities of Newport Beach and Laguna Beach have initiated a program in 2006 to protect and renovate sensitive marine life areas, areas of special biological significance (ASBS), adjacent to the cities' jurisdictions. The objective of this program is to identify and quantify those environmental impacts having the most deleterious effects on the water quality and habitat of the ASBSs and to prepare an Integrated Coastal Watershed Management Plan for these areas. These potential impacts may include constituent loading from dry weather and wet weather flows to the ASBSs, constituent loading from stormwater, public trampling and scavenging activities, fishing activities, cross contamination from tidal flows from Newport Bay, and other environmental stressors.

The cites contracted Weston Solutions, Inc. (WESTON®) as the lead environmental consulting firm to perform field testing at coastal regions near Buck Gully and a rocky intertidal zone at Little Corona del Mar. WESTON subcontracted Dr. Sunny Jiang at University of California, Irvine to perform bioassay of human viruses, coliphage, domoic acid, and *Vibrio spp.* in California mussels.

Introduction

Toxicity and bioaccumulation studies are the most definitive methods in which to assess contaminant-related impacts in the environment. In this study, bioaccumulation of contaminants of concern in California mussels was used to determine the integrated accumulation of bioavailable contaminants from various sources. Mussels are filter feeders that relay on collecting organic particles from large volume of water as food. A large number of bacteria and viruses has been found to accumulate in mussel tissue harvested from contaminated waterbody. Mussels are also known to bioaccumulate domoic acid, a neurotoxin produced by a diatom species in the ocean water. This biotoxin can be transported to seals and sea lions who use mussels as food source and cause morbidity and mortality in marine mammal population. In addition to the chemical contaminants that were analyzed by WESTON, a subcontract with University of California Irvine was made to test the concentration of bacteria (i.e., *Vibrio spp.*), viruses (i.e., coliphage and human adenovirus), and domoic acid in the mussels.

Materials and Methods

Mussels Samples—WESTON staff collected and delivered all mussel samples to Dr. Sunny Jiang's lab at University of California, Irvine. Table 1 shows a list of samples provided by WESTON and assays preformed by Dr. Sunny Jiang's lab. In the lab, ten mussels from each batch sample were opened using sterilized knife and scissors. Mussel tissues were removed carefully and transferred to a sterile container with PBS (pH 8.0) buffer. The mussel tissue was weighed and either used immediately for coliphage assay or stored frozen at -70 °C for nucleic acid extraction, PCR, and domoic acid assays.

Table 1. Summary of Mussel Samples Provided by WESTON and Bioassays Performed by UCI

Sampling Date	Description	Vibrio Enrichment	F-Coliphage Enrichment	Nucleic Acid Extraction	Domonic Acid Extraction
02/16/2007	Control	+	+	+	+
07/07/2007	Control	+	+	+	+
10/08/2007	Sample 1	+	+	+	+
10/08/2007	Sample 2	+	+	+	+
10/08/2007	Sample 3	+	+	+	+
10/08/2007	Sample 4	+	+	+	+
02/08/2008	Newport Harbor	+	+	+	+
02/08/2008	BG Intertidal	+	+	+	+

Sample Extraction—Mussel tissues were vortexed in PBS buffer for two mins. The mixture was then centrifuged briefly to settle the tissue debris, and supernatant was aspirated carefully to transfer into a fresh sterile tube. The supernatants were diluted using sterile PBS buffer for enrichment of F-coliphage or used directly for human adenovirus and domoic acid assays.

Enrichment for Vibrio spp.—A subportion of mussel tissue was used to enrich for *Vibrio spp.* using Alkaline Pepton Water (APW). Two 12.5 g of tissue were inoculated into each of sterile flask containing 25 mL of APW. The flasks were incubated overnight at 35°C. The next morning, 1 mL of the enrichment culture from each flask was collected and used for nucleic acid extraction and Polymerase Chain Reaction (PCR) detection of *Vibrio spp.*

PCR for Vibrio spp.—Total genomic DNA was extracted from the enrichment cultures using the following protocol. Cells in 1 mL of enrichment culture was harvested and resuspended in 500 uL of TE (Tris-EDTA pH 8.0) buffer. The cells were lysed using 10% SDS with proteinase K. Genomic DNA was extracted using CTAB-NaCl solution and purified using phenol/chloroform extraction. The DNA was precipitated using isopropanol and washed with 70% ethanol. The final DNA pellet was taken up in 30 uL DI water and 4 uL of each used for each PCR assay.

PCR primers and conditions for *V. cholerae* species are identical to those described by Jiang et al. (2001). These primers target 16S-23S intergenic spacer region of *V. cholerae* and detect both O1 and non-O1/O139 strains. *Vibiro vulnificus* species-specific primers are: vvf 5'- TTC CAA CTT CAA ACC GAA CTA TGA C -3' and vvr 5'- ATT CCA GTC GAT GCG AAT ACG TTG -3' to yield an amplicon of 205 bp. The following PCR conditions are used: 94°C for three min for initial denaturation followed by 30 cycles of 94°C for one min, 65°C for one min, 72°C for one min, and a final extension at 72°C for five min. The primers for *Vibrio parahaemolyticus* species-specific primers target *tlh* gene. They are *tlh*F 5' – AAA GCG GAT TAT GCA GAA GCA CTG- 3' and *tlh*R 5'- GCT ACT TTC TAG CAT TTT CTC TGC-3' to yield an amplicon of 450 bp. The PCR conditions are identical to that for *Vibrio vulnificus*. To detect toxin gene in *Vibrio parahaemolyticus*, primers targeting *tdh* gene are used. They are *tdh*F 5' – GTA AAG

GTC TCT GAC TTT TGG AC -3' and *tdh*R 5' – TGG AAT AGA ACC TTC ATC TTC ACC -3' to yield an amplicon of 269 bp. The PCR condition for *tdh* was identical as for *tlh* gene but with a lower annealing temperature at 55°C. All PCR results were visualized by gel electrophoresis. Positive and negative controls were included in each set of experiment. Only results passing quality assurance (QA) are included in the report.

F-Coliphage Assay by Two-Step Culture Enrichment—The EPA protocol 1601 was used for assay of F-coliphage in mussel tissues. In brief, mussel tissue extracts were serially diluted and inoculated into nutrient broth with *E.coli* F-amp host amended with antibiotics. The mixture was incubated overnight at 37°C. The next morning *E. coli* bacterial lawn was prepared, and one drop of the supernatant from each enrichment culture was spotted onto the lawn. After overnight incubation, the plates were examined for clearing spots. The clearing of the test spot was scored positive for F-coliphage and the absence of clearing was scored as negative.

Human Adenovirus Detection by PCR—Mussel tissue extracts were used for viral nucleic acid purification. Briefly, the mussel extract was cleared for large debris by low-speed centrifugation. The supernatants were used for nucleic acid extraction using Qiagen Viral purification kit. The final nucleic acid extract was eluded in TE buffer. Human adenovirus-specific primers were used for PCR amplication of the target using PCR protocol as reported by Xu et al. (2001). The PCR amplicons were assayed by gel electrophoresis.

Domoic Acid Assay—Mussel tissue extracts were purified by filtering through 0.45 µm pore-size filters to remove debris. The concentration of domoic acid was determined by a colorimetric immunoassay using Domoic Acid Test Kit (Mercury Science Inc.) following manufacturer's protocol. The assay is developed by the Center for Coastal Fisheries and Habitat with proven reproducibility and sensitivity. A replicate of 50 ul for each sample was used for testing in a microtiter plate. The samples were incubated with domoic acid tracer solution and washed three times. The substrate solution was then added for color development. The absorbance at 450 nm wave-length was read using microtiterplate reader.

Quality Assurance and Quality Control Procedures—QA and quality control (QC) of laboratory reagents and instruments was performed before sample processing and analyses. For nucleic acid extraction and purification, all reagents, test tubes, and pippettes were tested independently three to five times to ensure the accuracy and to ensure they were free of contamination of the target organisms. At least one negative control was included during each extraction to ensure no cross-contamination during extraction procedure. For PCR analysis, all assays were performed in a designated PCR hood. The working area was wiped with 1% bleach followed by 75% ethanol and radiated by UV radiation for 15 mins before each PCR set up. All PCR reagents, enzymes, instrument, pippettes, and tubes were tested independently three to five times to ensure of accuracy and free contaminants. At least one positive and one negative control were included in each PCR run to control for cross-contamination and false negative during PCR procedure. PCR amplicons were analyzed in a separate room to prevent

amplicon contamination (PCR tubes were opened and disposed only in the gel room). Assays that did not satisfy this QA/QC requirement were rejected, and the test was repeated.

For *Vibrio spp.* and F-specific phage isolations, culture medium, and sterile petri-dishes were purchased from certified commercial venders. All instruments, incubators, and reagents were tested before used for the assay. All antibiotics were purchased from certified commercial venders. The positive and negative results were verified by multiple researchers in the lab. All raw data were reviewed by principal investigator in the lab periodically.

Results and Discussion

Vibrio spp. are the most commonly found bacteria in coastal environments. However, several *Vibrio spp.* are also opportunistic pathogens to human. *Vibrio cholerae,* the causative agent of disease cholera, is responsible for seven pandemics in the world. In the United States where drinking water and sanitation systems are well developed, the diseases related to *Vibrio spp.* are often attributed to consumption of shellfish contaminated with high concentration of *Vibrio parahemolyticus* and *Vibrio vulnificus.* It is also important to note that although *V. choelrae*, *V. parahaemolyticus*, and *V. vulnificus* are wide spread in the coastal water, only the subspecies that contain toxin genes will cause severe illness in human. Low concentrations of *Vibrio spp.* are commonly found in shellfish tissue. Increased concentrations of *Vibrio spp.* in shellfish are detected when coastal waters are loaded with nutrients from land runoff.

Three types *Vibrio spp.* were tested in the mussel samples collected by WESTON. Table 2 shows that *V. vulnificus* was absent in all samples. Non-toxin *V. cholerae* was found in two mussel samples collected on October 8, 2007. *V. parahaemolyticus* was the most frequently detected species among mussel samples. A positive *V. parahaemolyticus* result was also found in the control mussel sample prior to the deployment into the coastal ocean. This control sample was also the only sample containing *tdh* toxin gene.

Table 2. Detection of *Vibrio spp*. in Mussel Tissue Samples by Polymerase Chain Reaction

Sampling Date	Description	*Vibrio cholerae (16-23int)*	*Vibrio parahaemolyticus (tlh)*	*Vibrio parahaemolyticus toxin (tdh)*	*Vibrio vulnificus (vvh)*
02/16/2007	Control	-	+	+	-
07/07/2007	Control	-	-	-	-
10/08/2007	Sample 1	-	+	-	-
10/08/2007	Sample 2	-	+	-	-
10/08/2007	Sample 3	+	+	-	-
10/08/2007	Sample 4	+	-	-	-
02/08/2008	Newport Harbor	-	+	-	-
02/08/2008	BG Intertidal	-	-	-	-

Coliphages are a group virus infecting coliform bacteria. F-specific coliphage has the morphology similar to human poliovirus and behaves similarly to polio in terms of disinfection characteristics during sewage treatment process. F-coliphage is currently considered by the United States Environmental Protection Agency (USEPA) as an indicator for human virus contamination. The results of this study show (Table 3) that F-coliphage was only present in one of the samples taken on October 8, 2007. This suggests that fecal contamination is not a problem in most portion of the coastal region.

Table 3. Detection of F-coliphage in Mussel Tissue Samples by Multiple Tubes Culture Enrichment

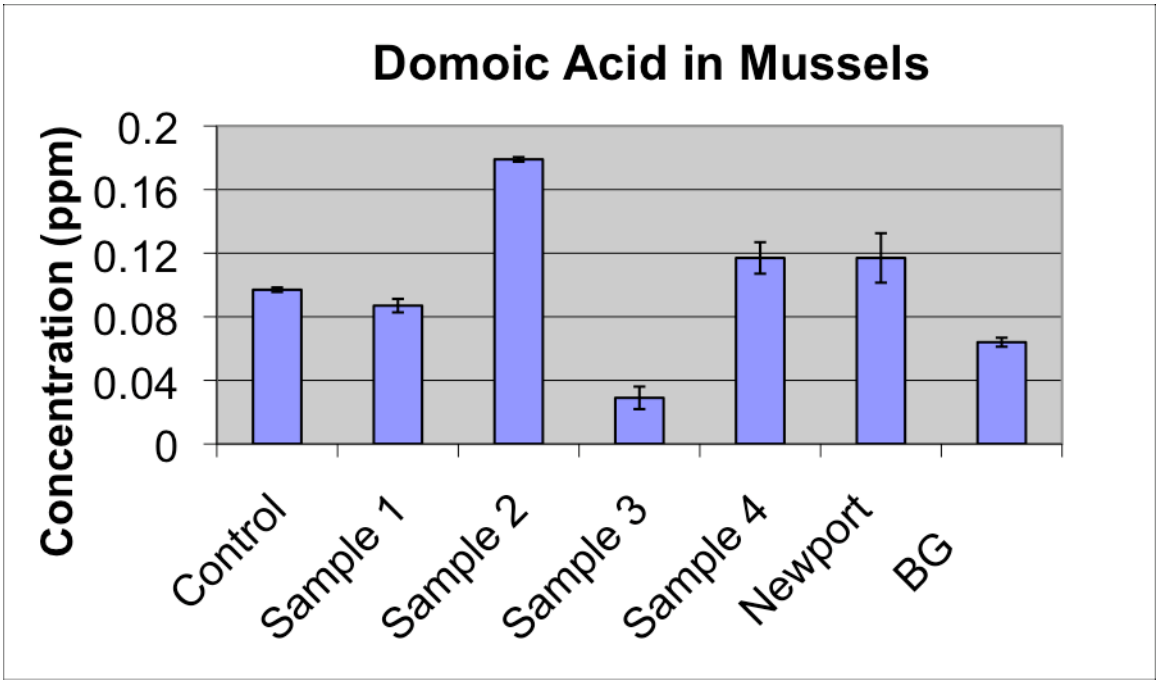
Sampling Date	Description	F-Coliphage Enrichment		
		10 mL	10 mL	10 mL
02/16/2007	Control	-	-	-
07/07/2007	Control	-	-	-
10/08/2007	Sample 1	-	-	-
10/08/2007	Sample 2	-	-	-
10/08/2007	Sample 3	-	-	-
10/08/2007	Sample 4	-	-	-
02/08/2008	Newport Harbor	+	-	-
02/08/2008	BG Intertidal	-	-	-

Human adenoviruses contain 51 serotypes. Only serotypes 5, 40, and 41 cause childhood diarrhea. Other serotypes are responsible for respiratory illness. Most people are infected by adenovirus during childhood, which may or may not display any symptoms, but then carry long-term immunity against the viruses. Human adenoviruses are frequently found in sewage and are used as a marker for human sewage contamination in the environment. The results of the mussel testing (Table 4) show that human adenoviruses are detected in one of the samples collected on October 8, 2007, suggesting contamination of trace amount of human waste in the area prior to the sample collection. It should be noted that the test examines the genetic material of adenovirus but provides no information on the infectivity of the virus.

Table 4. Detection of Human Adenoviruses in Mussel Tissue Samples by Ploymerase Chain Reaction

Sampling Date	Description	Adenoviruses
02/16/2007	Control	-
07/07/2007	Control	-
10/08/2007	Sample 1	-
10/08/2007	Sample 2	-
10/08/2007	Sample 3	+
10/08/2007	Sample 4	-
02/08/2008	Newport Harbor	-
02/08/2008	BG Intertidal	-

Domoic acid is a toxin produced by diatom *Pseudo-nitzschia*. In the event of *Pseudo-nitzschia* bloom in the coastal water, the commercial shellfish harvestings are closed for human health protection. However, a low dose of domoic acid is commonly found among shellfish samples. The results of domoic acid testing (Figure 1) show that domoic acid concentrations in all samples are less than 0.2 ppm which are significantly lower than California Department of Public Health standard. Domic acid concentration can reach up to 160 ppm in shellfish during a bloom event.



Conclusions

1.*Vibrio spp.* are infrequently detected in the mussel samples suggesting the area of the coast is not subject to abnormal pollution from land runoff.

2. Coliphage and adenoviruses are also rarely detected in mussel samples. Two of the sampling sites (Newport Harbor and Site 3) may have low impact from fecal waste contamination.

3. Domoic acid in the mussel samples is significantly lower than the State Standard suggesting the area of the coast is not impacted by harmful algal bloom.

References

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