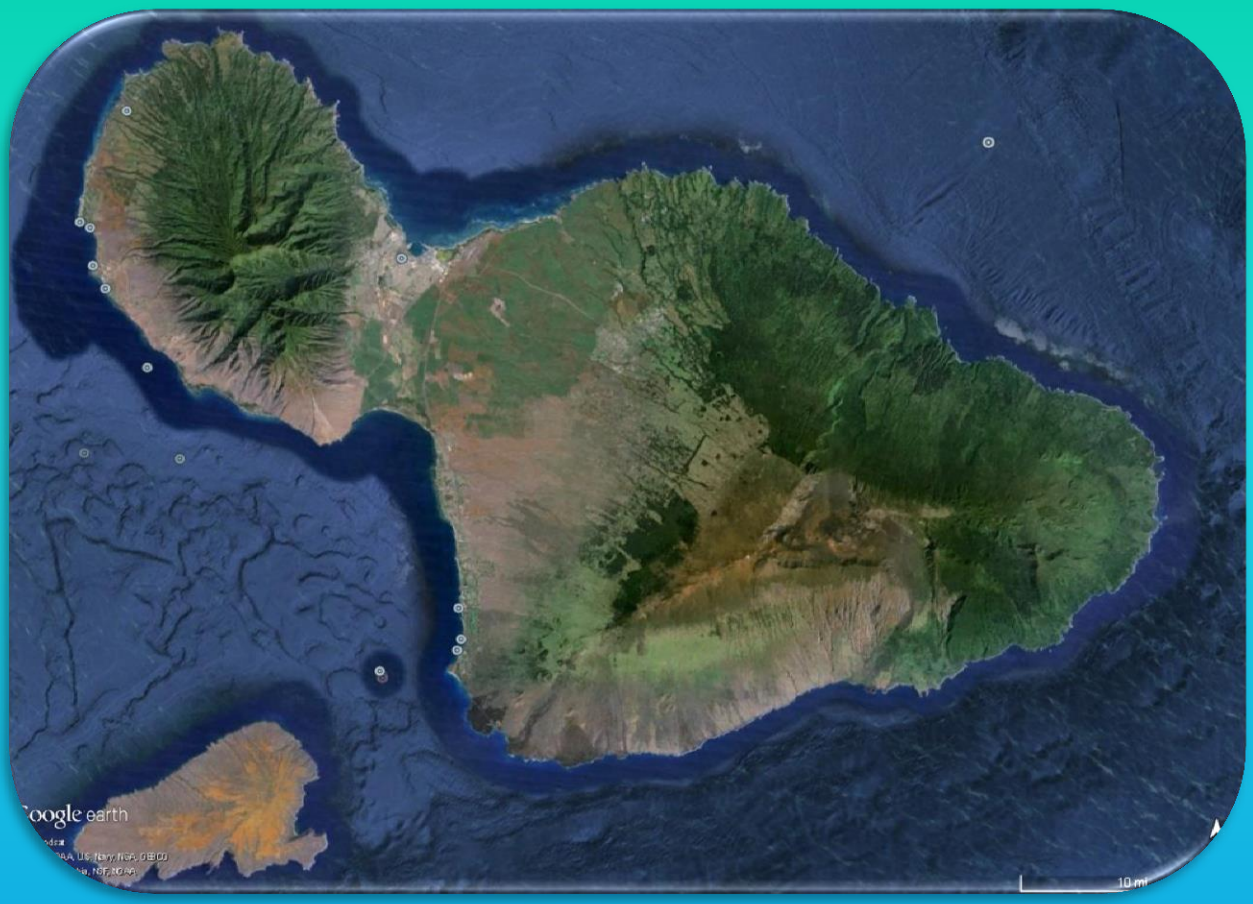

Environmental Investigation into impacts of LBSP on Coral Health in West Maui, Hawai‘i

CRCP Project 502 Final Report

West Maui, HI has been plagued with reports of poor water quality in the nearshore coastal zone, fecal indicators exceeding EPA standards, and algal blooms for over 20 years with a corresponding steady decline in coral covery from 70% (1990s) to 27% (2006). This final report provides baseline data related to bacterial water quality in wet and dry seasons and toxicity bioassay data at multiple locations along the Maui coastline from sediment and water sampled in 2012 and 2013. These data will help clarify the role of wastewater injection wells may play in coral decline and assist in best management practices for monitoring efforts. This information can help strategically focus costly management efforts on the greatest risk factors for mitigation and restoration of these vulnerable marine resources.

Final Report
CRCP Project 502

Environmental Investigation into Impacts of LBSP on Coral Health in West Maui, Hawai‘i

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August 1, 2016

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Executive Summary

Resource managers are faced with an ever increasing number and magnitude of threats from coastal development, tourism and new industries, particularly as these and other threats expand into previously untouched areas. The goals of this project were: 1) to determine if localized anthropogenic pollutants, including sewage, are impacting specific bays along Maui's west coast and 2) to provide practical training and technology transfer to local resource managers and students in two simple methods to initiate anthropogenic threat assessments. This final report contains the results of a two year scientific study (2012-2013) concentrated in the Ka'anapali watershed, a Coral Reef Task Force's (CRTF) priority site. Kahekili Beach Park and nearby waters were the focus of sampling efforts as well as an extended survey of other embayments along Maui's south and west coastlines. A description of the training course is also included in this report.

Two approaches were used to assess anthropogenic inputs and their possible impacts to the coastal waters of Maui Hawai'i. Bacterial fecal-indicators (*Enterococcus*, fecal coliforms) of sewage input and other potential human (salt tolerant *Staphylococcus* sp. and *Staphylococcus aureus)* and coral *(Serratia marcescens)* pathogens were cultured on selective media and enumerated from water column samples. Sediment porewater toxicity tests were conducted using the sea urchin embryo development assay as an indicator of anthropogenic pollutants. Together these data were used to identify sites that pose the highest risk to coral health.

Ka'anapali Priority Area Results

Bacterial Water Quality

Water samples from thirteen sites within the Ka'anapali watershed in proximity to Kahekili were evaluated for bacterial contamination, eight sites in September 2012 and five sites and in February 2013 (dry and rainy seasons, respectively). In the dry season, none of the sites exceeded the EPA limit for the fecal indicator, *Enterococcus* (104 colony forming units (cfu)/100 mL, single sample). Site WLA (southernmost dry season sampling site) had the highest levels of *Enterococcus*, fecal coliforms and *Serratia marcescens*. *Staphylococcus* also was elevated on the south end of the sampling area during the dry season (sites WLA and WDB) compared to other sites. In the rainy season, two sites, Boneyard and Runway exceeded the EPA single sample water quality limit for *Enterococcus*. A sample from Sand Channel (near site WLA on the south end of Kahekili) had the highest levels of fecal coliforms and *Serratia marcescens* during the wet season. *Staphylococcus* levels in February 2013 were similar at all sites tested, but 10-fold higher than observed during the dry season.

Sediment Porewater and Seep Water Toxicity

Sediment porewater toxicity testing using sea urchin development was conducted on six samples obtained in September 2012 near Kahekili. Three samples tested positive for toxicity in the sea urchin development assay that were significantly different from the control. The WLA site demonstrated the most severe toxicity with only 13% normal embryo development, while WDB and South of the South Seeps displayed 53% and 52% normal embryo development, respectively. Analytical chemical analysis of South of South Seep porewater detected pharmaceutical compounds and the herbicide, atrazine. Analysis of South Seep water detected one pharmaceutical compound, carbamazepine, and two herbicides, atrazine and simazine. Sediment porewater was sampled again on June 25, 2013 from four sites (Runway, South Seep, Bone Yard and Sand Channel) near Kahekili Beach Park and in the general vicinity of those sampled in 2012. Two additional sites north and south of the Kahekili Beach Park (Honokowai Point and Black Rock) were also sampled. Black Rock showed no toxicity while the other five samples showed 100% toxicity (i.e., no normal embryo development).

Conclusions

Analysis of water from the South Seep at Kahekili indicated that it was not a source of area bacterial contamination. The proximity of the Kahekili Beach Park to areas with high levels of fecal indicator and other bacteria compared to areas with similar numbers of beach-goers with low fecal indicator levels suggest an alternative source maybe compromised sewer lines in this area. Recent discussions with Maui County officials indicate they recognize the problem and are actively planning to upgrade the sewer system. These actions should assist in improving marine water quality. Higher levels of human-associated bacteria during the wet season (winter) also could result from increased recreational water use and increased storm runoff. Maui reportedly has one of the highest levels of staph infections in the U.S.; however, since there are no bacterial water quality standards for *Staphylococcus* or *S. aureus,* levels are not monitored. Identifying sources, understanding the epidemiology and disease dynamics of staph infections are important areas for future public health research as well as determining if they play a role in affecting coral health.

The source of sediment porewater toxicants was not determined in this study. The presence of pharmaceuticals is indicative of sewage wastewater. Simazine is a common landscaping herbicide which could enter the reef environment through runoff or possibly waste water. Atrazine is a crop-protection herbicide that is not commonly used along the Kahekili coastline and its detection in this and other studies suggests a possible route though groundwater contamination. Knowing that these compounds exist in waters off West Maui is the first step in determining their risk to coral health. An important next step is to determine concentrations that cause adverse effects on coral health. This information would allow resource managers to set water quality criteria for these compounds in coral reef habitats.

Maui Coastline Evaluation

Bacterial Water Quality

Bacterial water quality was surveyed along the Maui coastline from Honolua Bay to La Perouse Bay by enumerating fecal indicator bacteria and other potential human and coral pathogens during the dry (9 sites) and wet (9 sites) seasons. During the dry season none of the surveyed sites exceeded the EPA bacteria water quality criteria for *Enterococcus*. Napili Bay had the highest dry season levels of fecal coliforms, *Serratia marcescens* and *Staphylococcus.* During the rainy season, both samples taken from Kapalua Bay exceeded the single sample EPA water quality standards for *Enterococcus*. Wet season total *Staphylococcus* levels exceeded 14,000 cfu/100 mL at Honokeana, Napili and Kapalua. Kapalua Bay also had the highest numbers of fecal coliforms, while *S. marcescens* was prevalent at Napili Bay during the wet season. Follow-up sampling in Kapalua Bay (May 2015, early dry season) for *Enterococcus* and *Staphylococcus* resulted in no detectable *Enterococcus* while staph levels of ~2000 cfu/100 mL were double the levels observed in September 2012 (late dry season) at Kapalua.

Sediment Porewater Toxicity

Sediment samples were collected in September 2012 from 15 sites along the Maui coastline that ranged from Kapalua Bay to La Perouse Bay and from six sites in 2013 from Honolua Bay to La Perouse. Sediment porewater (2012, 2013) was tested for toxicity using the sea urchin embryo development bioassay and a coral cell mortality assay (2012). Porewater analyzed with the sea urchin development assay in 2012, exhibited high levels of toxicity from the Pier at Kalaepohaku, Lahaina, Honokeana Cove and north and south sides of Kapalua Bay. Lipoa Place, Kahana Bay and Napili Bay showed moderate levels of toxicity. Four sites with positive toxicity (north & south sides of Kapalua Bay, Pier at Kalaepohaku and Lipoa Place) were tested in a coral cell mortality assay. Each sample displayed similar toxicity as with sea urchin embryo development. The south side of Kapalua Bay was the only 2013 site of the 6 tested that showed significant toxicity with 35% normally developed embryos.

Toxicity Identification Evaluations (TIEs) using sea urchin (SU) embryos were conducted in 2012 on 7 samples that tested positive in the porewater assay: Kapalua Bay, Honokeana Bay, Olowalu North, Olowalu South, the Pier at Kalaepohaku, Lipoa Place and Kahekili South Seep water. The results indicated that sediment porewater toxicants were predominantly associated with organically derived pollutants. Lipoa Place results suggest that metals may also be a contributor to the toxicity observed in this sample.

Conclusions

Results of the bacterial water quality analysis showed that Kapalua, Napili and Honokeana Cove embayments along the West Maui coastline may be impacted by sewage and/or storm drainage overflows. Sewer repairs that were undertaken at Kapalua in 2014-2015 appear to have contributed to improved water quality by reducing sewage input. However, Napili Bay and Honokeana Cove also may need sewer line evaluations/renovations.

South Maui sampling sites (Ulua Beach Park, Wailea-Polo Beach, Makena Park's Little Beach, Makena Road and La Perouse) all tested negative for toxicity, indicating a low risk for adverse effects on coral health.

West Maui and South West Maui sampling sites tested positive in 2012 for toxicity, indicating a high risk for adverse effects on coral health. The study results also suggest that anthropogenic pollutants are likely significant risk factors contributing to coral reef degradation in many bays along the Maui coastline. Preliminary evaluations indicate that each bay appears to have its own complement of stressors and/or pollutants. Therefore each will require their own investigation to determine specific causes of impairment or degradation, and customized mitigation actions will be needed to support either passive or active restoration activities.

Training Class

To aid resource managers in prioritizing potential impacts from human activities, the Coral Disease and Health Consortium (CDHC) provided training in: ***Practical Methods for Conducting Threat Assessments for Reef Managers.*** Eighteen participants representing local NGOs, and state and federal resource managers attended the three day workshop, September 25-27, 2012, hosted by Humpback Whale National Marine Sanctuary in Kihei, Maui, Hawai'i. Participants received classroom instruction on risk (threat) assessment and hands-on, practical application of these concepts using a local case study along the coast of Kahekili, West Maui. The field excursion and laboratory activities focused on training in proper field sampling techniques and laboratory methods used to conduct bacterial water quality testing and sea urchin development porewater toxicity assays.

INTRODUCTION

West Maui, HI has been plagued with reports of poor water quality in the near shore coastal zone for over 20 years (HIDOH 2012). Fecal indicators have exceeded EPA standards, and algal blooms have been prevalent. The Coral Reef Assessment and Monitoring Program (CRAMP) reports that there has been up to 67% live coral cover loss in their long-term monitoring sites (Ross *et al.* 2012). A corresponding steady decline in coral cover at Kahekili from 55% (1990s) to 33% (2006) has also been reported (Williams *et al.* 2008). Much attention has been focused on the sewage treatment plants and the practice of using injection wells in this region to dispose of treated wastewater. A 2009 USGS study (Hunt and Rosa 2009) provided evidence of a plume, with nearshore freshwater springs containing a plethora of pharmaceuticals and personal care products. Effluent escaping into coastal waters was further supported by an extensive nitrogen isotope (sewage marker) survey throughout Maui. Injected effluent from sewage plants was named as a possible source of the elevated nitrogen levels in the Kahekili area (Dailer *et al.* 2010). Others argue that sediment runoff, natural terrestrial nitrogen sources, fertilizer and legacy agro-chemicals are responsible for algal blooms and coral decline (U.S. EPA, Wendy Wiltse, personal communication). Though there is compelling evidence for injection wells causing environmental hazards for public recreational activities and coral health, it is insufficient to clearly link the practice of injection well disposal of treated sewage water as having detrimental effects on coral health.

The purpose of this study was to determine if localized anthropogenic pollutants are impacting the Kahekili area and other bays along Maui's west coast. The information obtain from this project contributes to establishing baseline data for tracking change after implementing Best Management Practices (BMP). For example, this baseline information can assist in determining the efficacy of sewage treatment system upgrades when they are implemented in West Maui. This information is being shared with NOAA and USACE to inform their watershed management planning and provide Federal (EPA) and state (HDLNR) decision-makers more definitive answers before undertaking costly wastewater improvements to appropriately regulate the wastewater discharge, and manage other pollution sources at the site.

The project objectives were to:

1) Survey West Maui bays for levels and identity of fecal-associated bacteria and other pathogens in dry and rainy seasons, as indicators of sewage contamination;

2) Assess the potential toxicity of sediment porewaters from Kahekili and surrounding bays; and

3) Provide practical training and technology transfer in two methods used in threat assessments to local resource managers and students.

BACTERIAL WATER QUALITY TESTING

Objective 1. Conduct Bacterial Water Quality Surveys in Kahekili and Other Maui Bays

I. BACKGROUND

A. U.S. EPA Bacterial Water Quality Standards

The Clean Water Act (CWA) of 1972 established the regulatory framework for controlling pollutant discharges into U.S. waters and establishes water quality standards for surface waters. It was amended in 1977 (Clean Water Act of 1977) and 1987 (Water Quality Act of 1987). The Safe Drinking Water Act of 1974 (SDWA, with amendments in 1986 and 1996) also regulates water quality from chemical and biological pollutants. The EPA approves sampling procedures and analytical methods used to determine chemical, microbiological and radiological components in wastewater under the CWA as well as methods for drinking water contaminants under the SDWA. These regulations also extend to marine and freshwater recreational waters and with associated biological water quality criteria.

Coliform bacteria are used as indicators of fecal contamination in water because they are associated with normal intestinal bacteria but in much higher concentrations than the microbial pathogens that are potentially present. Prior to 1986, bacterial water quality testing was an index based on the ratio of total coliforms to fecal coliforms with recommended maximum densities not to exceed geometric means of 200 organisms per 100 ml in recreational waters (U.S. EPA 1986). In 1986, the criteria were updated to reflect bacteria that were of fecal origin as a better indicator of fecal contamination. The primary indicators of fecal contamination included fecal coliforms, enterococci and *Escherichia coli (E. coli)*. Fecal coliforms are also called heat-tolerant or thermotolerant coliforms due to the fact that these bacteria live primarily in warm-blooded animals. Epidemiological studies found that enterococci and *E. coli* had a higher degree of association with outbreaks of certain diseases than fecal coliform counts (U.S. EPA 1986). Fecal coliforms and *E. coli* are used as fecal indicators for shellfish harvesting waters while Enterococcus concentrations are used for marine recreational waters. Thus enterococcus limits (35cfu/100 mL, geometric mean; 104cfu/100 mL single sample maximum allowable density) were recommended in the 1986 *Ambient Water Quality Criteria for Bacteria* (U.S. EPA 1986, 2003). The Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000 required each state and territory with coastal recreational waters to adopt bacterial water quality criteria that are as protective of human health as those proposed in 1986 (U.S. EPA 2004). In 2012, EPA released new recreational water quality criteria (U.S. EPA 2012a) that provides two sets of threshold concentrations single sample limits and geometric mean over a 5 days within a 30 day period, representing magnitude, duration and frequency. As science evolves, new indicators and methods are being evaluated and updated by the EPA. The most recent addition is molecular testing using the quantitative polymerase chain reaction (qPCR) for enterococci (EPA Method 1611; U.S. EPA 2012b).

B. Hawai‘i Water Quality Standards

Hawai‘i Water Quality Standards are set forth in their public law HAR §11-54. Section 3(c) of the law defines three waterbody types (embayment, open coastal and oceanic) and uses a tiered classification

scheme of “AA” and “A” denoting use and protection of the water body (HIDOH 2012). Hawai‘i’s bacterial water quality criterion employs enterococci as a primary bacterial indicator of marine recreational water quality. Detection limits are set at a geometric mean of 35 colony-forming units (cfu)/100mL of water from a minimum of 5 samples within a 30 day period (which assesses the typical value of a set of samples) (SRG 2012), or a single sample maximum limit of 104 cfu/100mL.

Monitoring programs in Hawai‘i and other programs across the U.S. have found enterococci to be problematic as an indicator. This concern is supported by several studies showing that in tropical environments, *Enterococcus* can multiply outside of the human body (Byappanahalli and Fujioka 2004) as well as being found in feces of various wildlife species (e.g., feral mammals and birds). Findings by Byappanahalli and Fujioka (2004) describe the permissiveness of Hawai‘ian soils for the growth of *E. coli* and enterococci. They speculate that the exceedances of EPA standards in Hawai‘i streams could be due to run-off of these bacteria from the soil rather than sewage-borne pathogens during wet seasons in particular. Although not specified by the EPA, *Clostridium perfringens* was identified as an effective tracer of fecal contamination (HAR 2014) and is used by Hawai‘i Department of Health (HIDOH) as a second indicator to help confirm the likelihood of fecal contamination when enterococci levels exceed standard criteria limits.

C. Bacterial Water Quality Issues in West Maui

Impaired water quality is a significant problem in most coastal areas and a threat to the health of coral reefs of those in tropical and sub-tropical regions. In Maui County, the practice of shallow well injection of sewage treatment plant effluents and the subsequent seepage from these wells into the near shore environment (Hunt and Rosa 2009; Ross *et al.* 2012) have raised questions as to whether they may be contributing to the impaired water quality and decline in coral health, although there have been no findings of fecal indicators from seep water reported (SRG 2012). Aging and corroding cast iron sewer pipe leakage is also a growing problem for West Maui (Eagar 2011). Maui County officials recognize the issue (County of Maui 2011) and have undertaken a long-term rehabilitation program to correct ongoing system failures (County of Maui 2014). Livestock farming and feral animals also contribute to the bacterial load in Maui’s streams and runoff, particularly during wet seasons. *Staphylococcus aureus* infections and particularly infections that are methicillin resistant (MRSA) are also a growing concern for visitors and locals. Maui has been reported to have over twice the number of staph infections as those on the U.S. mainland (Fujimori 2007; Tummons 2010). Sewage and/or shedding of these bacteria by swimmers are also possible routes for *Staphylococcus* to enter the marine environment. Unfortunately there is no routine monitoring and water quality standards do not exist for *Staphylococcus* sp.

D. Bacterial Water Quality and Reef Health

West Maui, Hawai‘i has been plagued with steady declines in live coral and numerous reports of poor water quality (Brown 2008; Hunt and Rosa 2009; Dailer *et al.* 2010, 2012; Ross *et al*. 2012). Bacterial water quality for recreational waters (fresh and marine waters) are assessed using fecal bacteria as an indicator of the possible presence of pathogens in surface waters and for determining the risk of

disease. Though these standards are directed at human health safety, there is growing evidence that contaminated waters from sewage can carry pathogens that affect marine organisms (U.S. EPA 2003; 2004; 2012a). For example in Florida, fecal contamination has been traced to coral reefs (Lipp *et al.* 2002; Lipp and Griffin 2004); *Serratia marcescens* found in coral mucus has been identified as a causative agent acropora serratiosis (originally White Pox) for disease in *Acropora palmata* (Patterson *et al*. 2002; Sutherland 2003; Sutherland *et al*. 2010, 2011). Though the risk-potential for sewage associated pathogens affecting coral health is unknown, the linkage found between sewage and coral disease in the Caribbean warrants closer inspection in other coral reef habitats.

E. Strategy

The use of bacterial indicators as a strategy for determining possible sewage inputs in general or from freshwater seeps into the coastal waters of West Maui was accomplished by addressing the following:

1. What are the levels of sewage indicator bacteria in the coastal waters of Maui, with special emphasis along the coast of Kahekili, Maui, HI in dry vs. wet seasons?
2. Do fecal indicator bacterial densities exceed water quality standards (location vs season)?
3. What are levels of *Serratia marcescens* (linked to sewage and human and coral pathogenicity), in the coastal waters of Maui?
4. What are the levels of *Staphylococcus aureus* and total salt-tolerant *Staphylococcus* in coastal waters of Maui in dry vs wet seasons?

II. METHODS

A. Sampling Locations

1. Kahekili Sites

Sites within the Ka'anapali watershed, in proximity to Kahekili, (Table 1) were sampled at single time points for *Enterococcus*, fecal coliforms, *Staphylococcus* sp. and *Serratia marcescens*. Collection sites were chosen based on previous research and questions related to the contribution of wastewater injection wells and freshwater seeps to declines in coral health. The overall site selections in 2012 were made with guidance from Wendy Wiltse (U.S. EPA, Honolulu HI) that focused on two features. The first were the Kahekili 'live and dead zones' identified in work by Megan Ross and Paul Jokiel (2010). This work involved mapping Kahekili coral reefs showing 'live and dead zones' with varying degrees and types of degradation, primarily from algal overgrowth that increased in closer proximity to effluent inputs. The second were freshwater seeps thought to be associated with the use of wastewater injection wells that had been identified in work by Meghan Dailer's lab (2010, 2012) that characterized freshwater seeps and measured nutrients and algal blooms in the nearshore of Kahekili helped identify sites with seeps for sampling.

The six sites sampled in 2013 (Table 1) were selected on the advice of Dr. Darla White of the State of Hawai'i's Department of Land and Natural Resources (DLNR). Dr. White is a coral expert for DLNR and conducts regular monitoring activities in this area. Sites were selected based on knowledge of the freshwater seeps, history of the sites, proximity to 2012 sites, and coral and fish habitats of interest to DLNR.

Table 1. Coordinates for Bacterial Water Quality Sampling Sites along the Kahekili Nearshore.

Region (see map Fig. 1)	September 2012 Sampling Locations (Dry)	Latitude	Longitude	February 2013 Sampling Locations (Wet)	Latitude	Longitude
1	Kahekili Dead 1	20.939481	-156.69361	Runway	20.93954	- 156.69357
2	Kahekili Live 1	20.939111	- 156.69358	Healthy North	20.93909	- 156.69368
3	South Seeps	20.938568	- 156.69313	South Seeps	-	-
4	Weston Dead B (WDB)	20.937272	- 156.69374	Bone Yard	20.93734	- 156.69348
	Weston Dead A/ Dead 2 (WDA)	20.937269	- 156.69371	Weston Dead A/ Dead 2 (WDA)	-	-
	Weston Live B (WLB)	20.937062	- 156.69362	Weston Live B (WLB)	-	-
5	Sand Channel	-	-	Sand Channel	20.93624	- 156.69336
	Weston Live A (WLA) /Live 2	20.936240	- 156.69361	Healthy South	20.93681	- 156.69347

'-' indicates that the site was not sampled. Sites sampled in 2012 were selected by W. Wiltse of EPA to evaluate live vs dead zones. Sites in 2013 were selected by D. White of HDLNR based on their priorities. Note that though specific sites names differ, they have been organized by region to illustrate site proximities in the two years.

2. Other Maui Sampling Sites

In 2012, ten additional sites and in 2013 nine additional sites were sampled from Kapalua to La Perouse (Table 2) at single time points for *Enterococcus*, fecal coliforms, *Staphylococcus* sp. and *Serratia marcescens* in dry and wet seasons. Sites were selected based on advice and previous monitoring by Hawai'i Department of Health, volunteer citizen scientists of the Humpback Whale National Marine Sanctuary and embayments previously recognized with coral health impairment by DLNR monitoring and previous research on Maui by Dr. Craig Downs of Haereticus Environmental Laboratory (HEL). Selections were based on different types of land-use practices, beach parks with varying tourist visitation and resorts along with a low-visitation reference site (La Perouse). All locations were accessible from shore. Results showing pollutant inputs from sites that were sampled in 2012 and new issues arising (e.g., new development of Olowalu) were used to target 2013 sampling sites.

Table 2. Coordinates of Other Maui Sampling Sites for Bacterial Water Quality Analysis.

September 2012 Sampling Other Maui Locations	GPS Coordinates		February 2013 Sampling Other Maui Locations	GPS Coordinates	
Kapalua North	21.000369	- 156.666989	**Kapalua North**	21.00037	- 156.66699
Kapalua South	20.99895	- 156.66757	**Kapalua South**	20.99895	- 156.66757
Napili North	20.996619	- 156.666798	**Napili North**	20.99659	- 156.66672
Napili South	-	-	**Napili South**	20.99464	- 156.66748
Honokeana	20.991592	- 156.668678	**Honokeana**	20.99229	- 156.66890
Olowalu North	-	-	**Olowalu North**	20.80902	- 156.61353
Olowalu South	-	-	**Olowalu South**	20.80911	- 156.61133
Ma'alaea	20.792088	- 156.509978	**Ma'alaea**	-	-
Wahikuli Haycroft Park	20.796269	- 156.502878	**Wahikuli Haycroft Park**	-	-
Kalama Beach Park	20.730722	- 156.454015	**Kalama Beach Park**	-	-
Wailea Ulua Beach	20.691189	- 156.445238	**Wailea Ulua Beach**	-	-
Makena Rd (Reserve -Little Beach)	20.621762	- 156.439411	**Makena Rd**	20.61763	- 156.41592
La Perouse	20.590683	- 156.412983	**La Perouse**	20.59068	- 156.41298

'-' indicates that the site was not sampled.

B. Seawater Sample Collection

Prior to water collection sample bottles were sterilized with isopropyl alcohol, leaving residual alcohol in the bottles. At each site, sample handlers donned disposable nitrile gloves then swam to the appropriate site and while facing the current, placed a sterile 1 L screw-capped polypropylene bottle under water,

approximately 1 m below the surface. The bottle was opened, allowing it to fill with water. The container was sealed and brought to the surface and decanted downstream of the sampler. This process of rinsing the bottle with sample water was repeated three times prior to sample collection to remove residual isopropyl alcohol. The bottles of natural seawater (three per site) were sealed tightly, and placed in an ambient cooler filled with seawater for the trip to the laboratory (no more than 2 h).

C. Bacterial Recovery

Specific bacterial types were enumerated using the membrane filtration method (Britton and Greeson, 1987; Appendix I for detailed protocol). Well-mixed water volumes (1, 10 and 100 mL, triplicates) of each seawater sample were vacuum-filtered through nitrocellulose membrane filters (47 mm, 0.45 µm) and then placed onto 60 mm selective agar plates: DNase-toluidine blue-cephalothin (DTC, *Serratia marcescens*), mannitol salt agar (MSA, salt-tolerant *Staphylococcus* sp.), membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI, *Enterococcus* sp.) or fecal coliform selection (mFC) agar. Plates containing mEI, DTC and mFC agar were incubated at 42 °C and the MSA plates were incubated at 37 °C. Colony forming units (cfu) of the appropriate color (pink, *Staphylococcus epidermidis*; yellow, *Staphylococcus aureus*; white, undefined Staph species) were enumerated following 24-36 h incubations and results were normalized to 100 mL sample volume.

III. RESULTS & DISCUSSION

A. Bacterial Water Quality Findings near Kahekili, West Maui

A map of the Kahekili sampling area and sites with the highest bacterial load is presented in Figure 1. In September 2012 (dry season) none of the sites that were tested exceeded Hawai'i's single-sample bacterial water quality criteria (104 cfu/100 mL) *Enterococcus,* (Fig. 2; Table 3) or for fecal coliforms (Table 4). Levels of *Serratia marcescens* were also tested because it is associated with sewage, a potential human pathogen, and is associated with a Caribbean coral disease, acroporid serratiosis (aka white pox disease; Sutherland *et al*. 2010, 2011). This bacterium is an opportunistic pathogen with an unknown infectious dose for humans or coral. The concentration of *S. marcescens* at the WLA site was demonstrate to be 32.4 cfu/100 mL, the highest among all sites tested (Table 4). Total salt-tolerant *Staphylococcus and Staphylococcus aureus* were also enumerated (Table 5) because of concern over the high incidence of staph-related infections reported in Maui (HHIC 2006). Water samples from two sites, WDB and WLA, resulted in colonies that overcrowded the plates, resulting in too many colonies to count (TMTC). At other sites, counts ranged from approximately 5-300 cfu/100 mL for *Staphylocccus aureus*.

In February 2013 (wet season), two of the Kahekili sites (Bone Yard, Runway) exceeded the single sample water quality limit for *Enterococcus* (Figs. 1, 2; Table 3), but did not have a corresponding elevated level of fecal coliforms (Table 4). These differences are not unusual and may result from the permissiveness of their growth in Hawai'ian soils and run-off (Byappanahalli and Fujioka 2004). The low fecal coliform levels are not consistent with expected stormwater runoff and thus suggest an alternate source for the two Kahekili sites that had elevated levels of fecal indicators. The highest wet season level

of fecal coliforms was observed at Sand Channel, though lower than reportable levels. *Serratia marcescens* levels were highest at Healthy South and Sand Channel, both sites located on the southernmost section of the Kahekili sampling area (Fig. 1; Table 4). *Staphylococcus aureus* levels were 10-fold higher in the wet season than most sites tested during the dry season, and ranged from 785-1730 cfu/100 mL (Table 5). Although water quality standards do not exist for *Staphylococcus* or *Serratia*, these data can serve as baseline information for future investigations.

Figure 1. Map of Kahekili sampling sites in September 2012 (yellow numbers) and February 2013 (teal numbers), dry and wet seasons, respectively. Red diamond = sites exceeding single sample water quality criteria for *Enterococcus* (104 cfu/100 mL); green oval=sites with highest levels of fecal coliforms (none exceeded standards); blue square=sites with highest levels of *Serratia marcescens*; yellow triangle=sites with highest levels of *Staphylococcus* sp.

Table 3. Bacterial Enumeration of *Enterococcus* at Kahekili.

Region (see map Fig. 1)	September 2012 Sampling Locations (Dry)	*Enterococcus* cfu/100 mL	February 2013 Sampling Locations (Wet)	*Enterococcus* cfu/100 mL
1	Dead 1	0	Runway	151
2	Live 1	0	Healthy North	35
3	South Seeps	0	South Seeps	-
4	Weston Dead B (WDB)	0	Bone Yard	115
	Weston Dead A/ Dead 2 (WDA)	0	Weston Dead A/ Dead 2 (WDA)	-
	Weston Live B (WLB)	0	Weston Live B (WLB)	-
5	Sand Channel	-	Sand Channel	26
	Weston Live A (WLA) /Live 2	10.9	Healthy South	34.5

'-' indicates that the site was not sampled; cfu=colony forming units

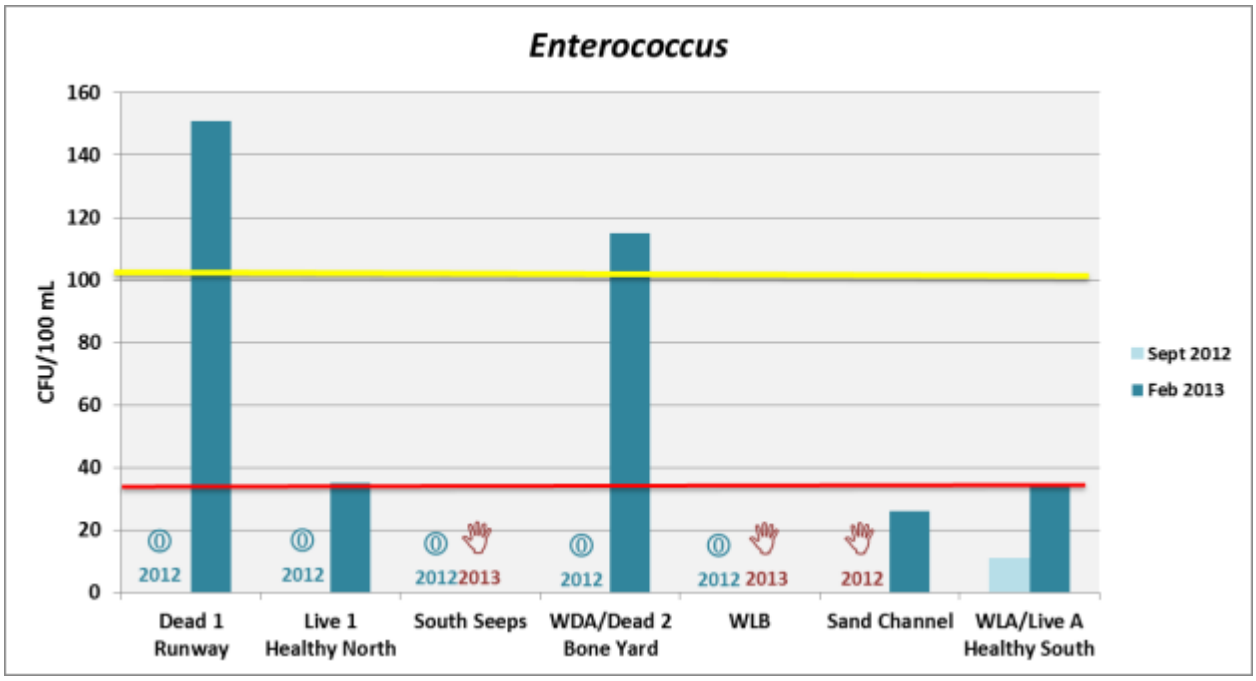

Figure 2. Levels of *Enterococcus* observed in dry (2012) vs wet (2013) seasons in sites near Kahekili. ⓪=none detected; ✋=site not sampled. Yellow line is the EPA single sample limit (104 cfu/100 ml). Red line is the EPA geometric mean standard (35 CFU/100 mL).

Table 4. Bacterial Enumeration of Fecal Coliforms and *Serratia marcescens* at Kahekili

Area N to S (Map Fig. 1)	Date	Location	Fecal coliforms (cfu/100mL) September 2012 (Dry)	Fecal coliforms (cfu/100mL) February 2013 (Wet)	*S. marcescens* (cfu/100mL) September 2012 (Dry)	*S. marcescens* (cfu/100mL) February 2013 (Wet)
1	9-23-12	Dead 1 Zone (class)	1.5	-	0	-
	1-28-13	Runway	-	8.5	-	1.5
2	9-23-12	Live 1 (class)	7.5	-	5.5	-
	1-28-13	Healthy North	-	22	-	1
3	9-18-12	South Seep	0	-	0	-
	9-23-12	South Seep (class)	0	-	0	-
4	9-18-12	Westin Dead Zone (WDB)	1.2	-	0	-
	9-23-12	Dead 2 Zone (class)	7.5	-	-	-
	1-28-13	Bone Yard	-	28.5	-	13.5
5	9-18-12	Westin Live Zone (WLA)	34.8	-	32.4	-
	9-23-12	Live 2 (class)	4	-	-	-
	1-28-13	Healthy South	-	16.5	-	26
	1-28-13	Sand Channel	-	64.5	-	29

'-' indicates that the site was not sampled; cfu = colony forming units

Table 5. Bacterial Enumeration of Total Salt-Tolerant *Staphylococcus* and *Staphyloccocus aureus* at Kahekili.

Area N to S (Map Fig. 1)	Date	Location	Total *Staphylococcus* (CFU/100mL) September 2012 (Dry)	Total *Staphyloccus* (CFU/100mL) February 2013 (Wet)	*S. aureus* (CFU/100mL) September 2012 (Dry)	*S.aureus* (CFU/100mL) February 2013 (Wet)
1	9-23-12	Dead 1 Zone (class)	295	-	255	-
	1-28-13	Runway	-	1620	-	1530
2	9-23-12	Live 1 (class)	260	-	200	-
	1-28-13	Healthy North	-	1750	-	1730
3	9-18-12	South Seep	10.6	-	4.8	-
	9-23-12	South Seep (class)	53.5	-	53.5	-
4	9-18-12	Westin Dead Zone (WDB)	TMTC	-	TMTC	-
	9-23-12	Dead 2 Zone (class)	100	-	50	-
	1-28-13	Bone Yard	-	1785	-	1065
5	9-18-12	Westin Live Zone (WLA)	TMTC	-	TMTC	-
	9-23-12	Live 2 (class)	130	-	90	-
	1-28-13	Healthy South	-	1495	-	785
	1-28-13	Sand Channel	-	1425	-	1165

TMTC: Too many to count; (-): not sampled; cfu = colony forming units

B. Bacterial Water Quality Findings for Other Sites along the Maui Coastline

Results of bacterial water quality testing along the Maui coastline are presented in Figure 3. In September 2012 (dry season) none of the 10 sites tested exceeded Hawai'i's single-sample bacterial water quality criteria for *Enterococcus,* (Fig. 4; Table 6) or fecal coliforms (Table 7). None of the sites demonstrated elevated levels of *Serratia marcescens* (Table 7). The north side of Napili Bay showed *Staphylococcus* sp. in excess of 14,000 cfu/100 mL (Table 8), with the other sites ranging from 260-7350 cfu/ 100 mL. The cause of these relatively high Staph levels is unknown, but may be linked to localized run-off, leaking sewer pipes or high numbers of beach-goers shedding these organisms. Interestingly, total *Staphylococcus* levels were substantially higher at Ma'alaea, Makena Rd and off the La Perouse parking lot, sites with higher numbers of recreational bathers, suggesting that contributions by swimmers may be a factor at these sites.

In February 2013 (wet season), North Kapalua Bay and South Kapalua Bay exceeded the single sample water quality criteria for *Enterococcus* (Table 6), while North Napili Bay did not surpass water quality standards. However, this level is sufficient to trigger action for follow-up sampling to determine if this is a trend that exceeded geometric mean water quality standards. Follow-up sampling in May 2015 showed no evidence of fecal contamination. During the time between 2013 and 2015, sewage lines in the area did undergo repair, in part from our Interim Report findings (The Voice Newsletter 2013; Maui County 2014). However, it is not clear whether the findings in May 2015 are a result of the beginning of the dry season, repair of sewage lines, or both. Neither fecal coliform nor *Serratia marcescens* wet season levels were elevated (Table 7) though interestingly S. marcescens levels were approximately and order of magnitude lower than those of the fecal coliforms. Realizing *Staphylococcus* levels were generally elevated in Maui waters, various dilutions were filtered before plating. The lowest dilution (1mL) tested generated such densities of colonies that plates were not countable yielding TMTC designations for Honokeana, Napili and Kapalua (Table 8). It is interesting to point out that most of the salt-tolerant Staph were *S. aureus*. Follow-up sampling in Kapalua Bay (May 2015, early dry season) generated levels that were approximately 2000 cfu/100 mL which is nearly double those measured in September 2012 during the late dry season. Increased recreational water use during the wet season (high tourist) likely contributed to higher loads of staph as well as more frequent runoff events.

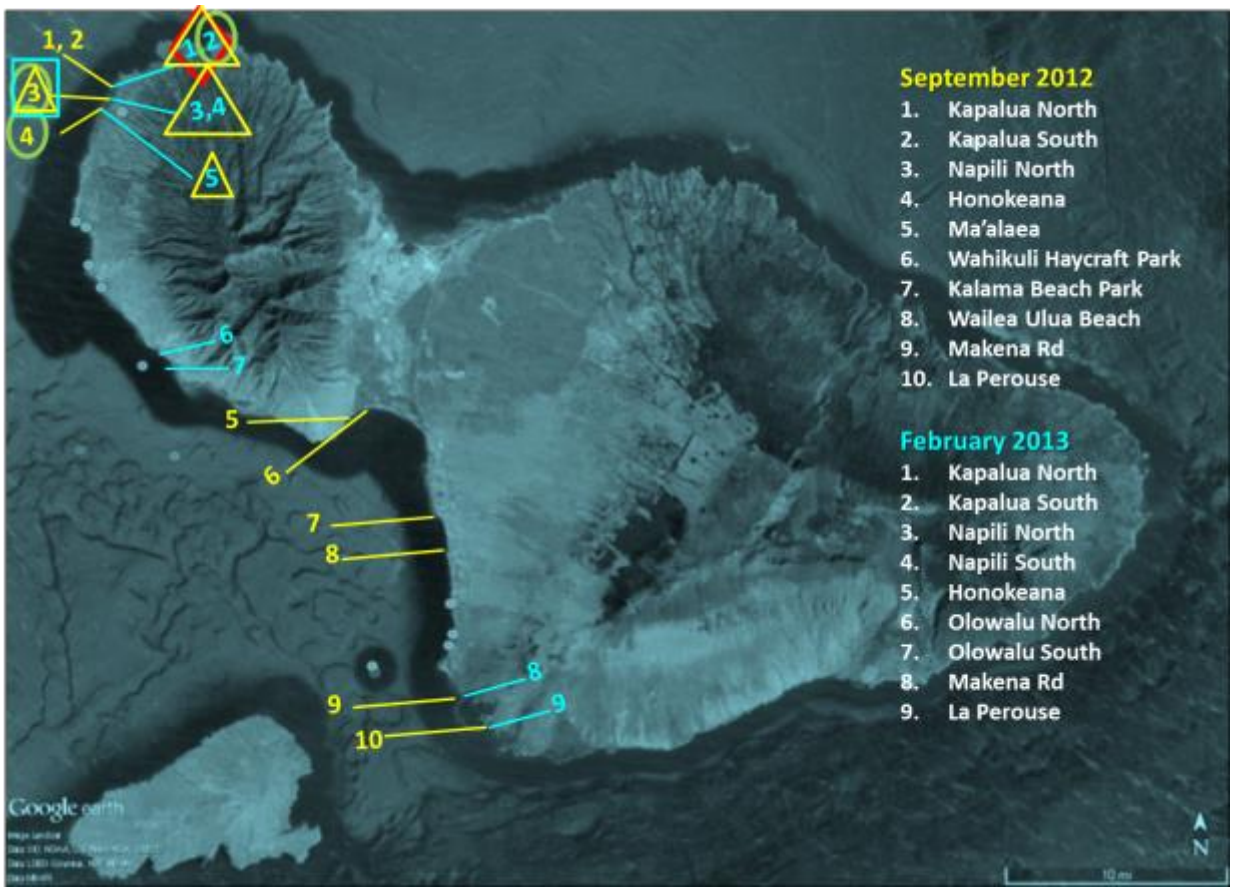

Figure 3. Map of bacterial water quality sampling sites along the Maui coastline in September 2012 (dry) and February 2013 (wet) seasons. Red diamond=sites exceeding one-time sampling criteria for *Enterococcus* (104 cfu/100 mL seawater); green oval=sites with highest levels of fecal coliforms (none exceeded standards); blue square=sites with highest levels of *Serratia marcescens*; yellow triangle=sites with highest levels of *Staphylococcus* sp. Tested bacterial species loads were highest at the northern end of West Maui.

Table 6. Bacterial Enumeration of *Enterococcus* in Dry and Wet Seasons along the Maui Coastline

Location	*Enterococcus* (cfu/100mL) September 2012 (Dry)	*Enterococcus* (cfu/100mL) February 2013 (Wet)
Kapalua North	0	166
Kapalua South	0	265
Napili North	9	97.5
Napili South	-	0
Honokeana	1.5	9.5
Olowalu North	-	27
Olowalu South	-	24.5
Ma'alaea	0	-
Wahikuli Haycroft Park	0	-
Kalama Beach Park	0	-
Wailea Ulua Beach	0	-
Makena Rd	0	29
La Perouse	0	1.5

'-' indicates that the site was not sampled; cfu= colony forming units. Red numbers indicate single sample exceedance of EPA water quality standard.

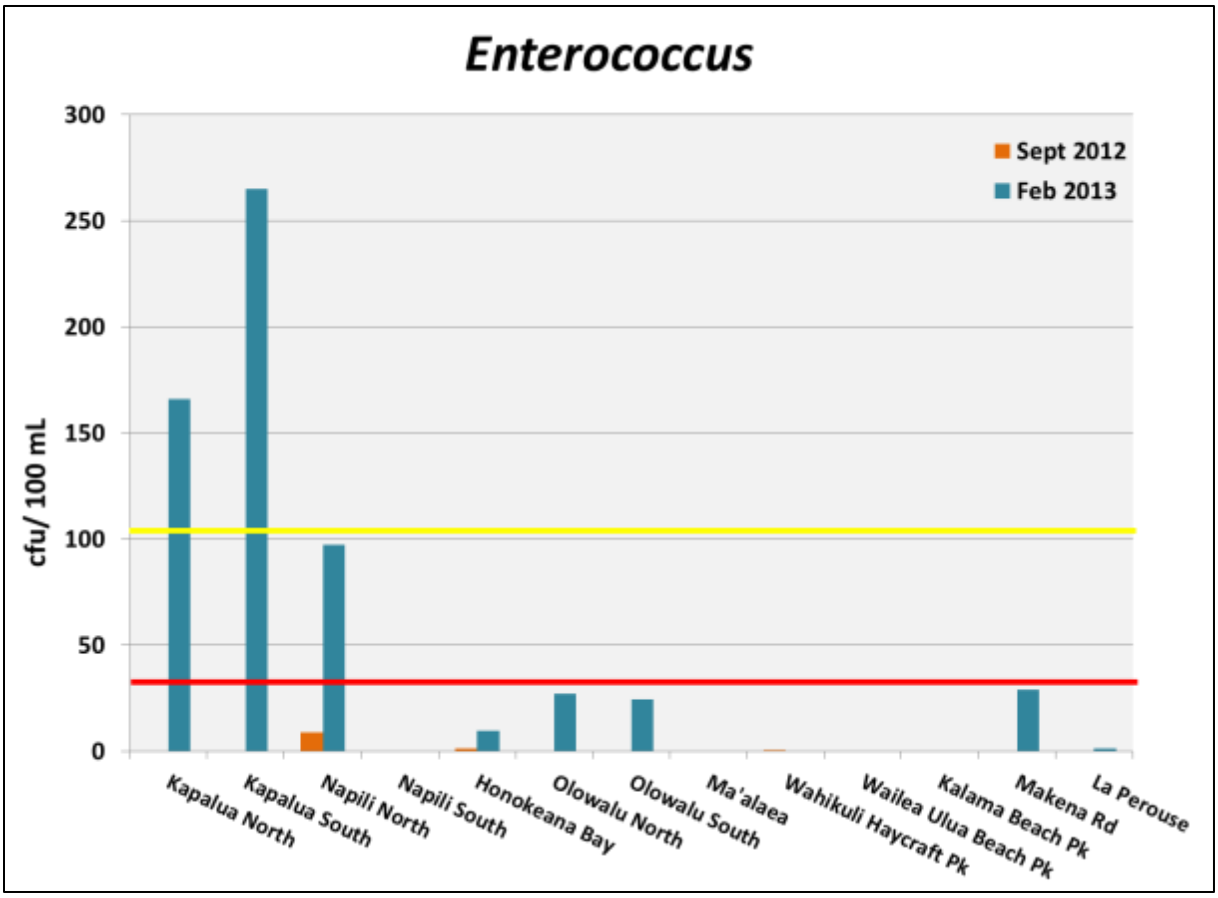

Figure 4. Levels of *Enterococcus* observed in dry (2012) vs wet (2013) seasons in other Maui coastal sites. Yellow line is the single sample limit (104 cfu/100 mL). Red line is the geometric mean standard (35 cfu/100 mL).

Table 7. Bacterial Enumeration of Fecal Coliforms and *Serratia marcescens* along the Maui coastline.

Location	Fecal coliforms (CFU/100mL) September 2012 (Dry)	Fecal coliforms (CFU/100mL) February 2013 (Wet)	*Serratia marcescens* (CFU/100mL) September 2012 (Dry)	*Serratia marcescens* (CFU/100mL) February 2013 (Wet)
Kapalua North	2	32.5	1	2.5
Kapalua South	3.5	11.5	2	1.5
Napili North	49.5	7.5	17.5	2.5
Napili South	-	25	-	2
Honokeana	44.5	11	2	1.5
Olowalu North	-	8	-	1
Olowalu South	-	5	-	3
Ma'alaea	2	-	-	-
Wahikuli Haycroft Park	9	-	-	-
Kalama Beach Park	1.5	-	0	-
Wailea Ulua Beach	0.5	-	0	-
Makena Rd	0.6	1	0	0
La Perouse (10 min walk)	2.2	9	1.5	0.5

'-' indicates that the site was not sampled; cfu = colony forming units

Table 8. Bacterial Enumeration of Total Salt-Tolerant *Staphylococcus* and *Staphyloccocus aureus* along the Maui Coast.

Location	Total *Staphylococcus* (cfu/100mL) September 2012 (Dry)	Total *Staphylococcus* (cfu/100mL) January 2013 (Wet)	*Staphylococcus aureus* (cfu/100mL) September 2012 (Dry)	*Staphylococcus aureus* (cfu/100mL) February 2013 (Wet)
Kapalua North	685	TMTC	675	TMTC
Kapalua South	995	TMTC	995	TMTC
Napili North	14000	TMTC	14000	TMTC
Napili South	-	TMTC	-	TMTC
Honokeana	605	TMTC	600	TMTC
Olowalu North	-	1137.5	-	1007
Olowalu South	-	815	-	735
Ma'alaea	1165	-	435	-
Wahikuli Haycroft Park	165	-	160	-
Kalama Beach Park	425	-	395	-
Wailea Ulua Beach	840	-	560	-
Makena Rd	1160	1350	540	1200
La Perouse 10 min walk	260	1290	135	1210
La Perouse Parking Lot	7350	-	3200	-

TMTC = too many to count; '-' indicates that the site was not sampled; cfu = colony forming units

IV. Summary of Bacterial Water Quality Testing

- *Enterococcus* levels were higher in the wet season (February 2013) than in the dry season (September 2012) in general across all sites tested.

- Kahekili sites, Bone Yard and Runway, showed single sample exceedances of Hawai'i water quality standards for *Enterococcus* (104 cfu/ 100 mL) during the wet season.

- Analysis of water from the South Seep at Kahekili showed no evidence of *Enterococcus,* fecal coliforms or *Serratia marcescens,* and very low levels of *Staphylococcus* sp. in the sample, indicating that it was not a source of any bacterial contamination in near-shore waters.

- Kapalua Bay (north and south sides) had single sample exceedances of *Enterococcus* during the wet season.

- Fecal coliforms were not elevated at any site tested in either season.

- There are no water quality standards for *Serratia marcescens.* However, WLA showed 10-fold higher levels in the dry season than other sites. In the wet season, Sand Channel and Healthy South (in close proximity to WLA) showed 5-10-fold higher levels than other Kahekili sites and 2-fold higher than Bone Yard.

- Maui has one of the highest incidences of staph infections in the U. S., however there are no water quality standards for this species of bacteria. Testing at Kahekili resulted in 10-fold higher staph levels in the wet season as compared to the dry season. Total salt-tolerant *Staphylococcus* and *Staphylococcus aureus* levels exceeded testing dilutions (TMTC) used in this study at Kapalua, Napili and Honokeana during the wet season. Levels were similar at other Maui sites in both wet and dry seasons. Increased recreational water use during the wet season (high tourist) may contribute to higher loads of staph as well as more frequent runoff events.

V. Conclusions

1. Taken together, the bacterial enumeration results indicate higher levels of fecal contaminated sewage in Maui waters in the wet vs dry seasons and point to possible sources as leaking sewer lines and/or higher numbers recreational bathers.
2. *Enterococcus* is used as an indicator of fecal pollution which can contain various pathogens, opportunistic pathogens, normal bacterial flora and accompanied by increased nutrient inputs, pharmaceuticals, personal care products, household chemicals and lawn care herbicides and pesticides. The presence of increased fecal pollution and/or raw sewage creates conditions for direct exposure to potential pathogens and/or indirectly can create a shift in bacterial communities associated

with coral leading, potentially leading to disease. Alternatively or in combination and depending on the pollutants present in sewage inputs, corals and other marine organisms may experience toxic conditions that increase susceptibility to opportunistic pathogens. The sites with high *Enterococcus* levels do not have elevated fecal coliforms which somewhat argues against stormwater runoff, as runoff is often the source of fecal coliforms. These two opposing pieces of data suggest the fecal contamination is more likely human origin such as sewage leakage or increased numbers of swimmers.

3. No fecal indicators were found from samples of freshwater seeps, thus the levels of *Enterococcus* and *Staphylococcus* spp. point to sources of untreated sewage.

4. *Serratia marcescens* is a sewage-associated bacterium that is an opportunistic pathogen for humans and has been associated with acropora serratiosis, a disease of Caribbean *Acropora palmata* coral. The levels found in Kahekili and Napili waters are of similar levels to those found in Florida Keys waters during outbreaks of acropora serratiosis. These higher levels of *S. marcescens* are indicative of the potential for coral disease.

5. *Staphylococcus* spp. and *Staphylococcus aureus* originate from farm animals as well as human sources including skin, raw sewage and treated sewage. The consistently high levels of *Staphylococcus* spp. and *S. aureus* in Maui waters indicate a continual input. While higher in the wet season which would suggest runoff as a likely source, the low levels of fecal coliforms suggest sewage and higher numbers of beach goers in the wet season may be more of a contributing factor.

6. Based on the results of this work next steps to consider are:
    a. Consider water quality monitoring to include *Staphylococcus aureus*, particularly due to the reported high incidence of methicillin-resistant *S. aureus* by establishing cause-effect relationships and transport and fate investigations.
    b. Conduct laboratory exposure studies to determine if waters with high fecal indicator levels are able to transmit disease.
    c. Investigate the impact of *S. aureus* on coral health.
    d. Promote public awareness when threat potentials are high for *Staphylococcus* spp. infections.

SEDIMENT TOXICITY TESTING

Objective 2. Assess the Toxicity of Sediment Porewaters off Kahekili and Other Maui Bays

I. BACKGROUND

Land-based sources of pollution (LBSP) are a major threat to the existence of coral reef ecosystems in the waters of Maui. The pollutants affecting Maui's coral reef ecosystems originate from relatively large, steep watersheds from multiple, diffuse sources. Land-use practices, whether agricultural or urban development, have resulted in a plethora of pollutants that have impaired the quality of the nearshore waters. The recent Wahikuli-Honokōwai Watershed Management Plan characterizes the watersheds of West Maui summarizing their current condition and those proposed for the future (SGR 2012). According to this report, non-point source pollutants predominate, and are being transported in surface and groundwater into the nearshore environment. Nutrients and sediment are regarded by water quality scientists (SGR 2012 and references therein) as the most problematic pollutants. However, Maui's nearshore environment is also plagued with legacy chemicals from the agricultural industry (i.e., sugar cane, pineapple) as well as current use herbicides, pesticides and fertilizers as crops change in agricultural areas. Urban areas contribute chemicals from wastewater effluents and injection well seeps (e.g., pharmaceuticals, personal care products, nutrients), landscaping activities (e.g., fertilizers, herbicides, pesticides). Aggressive commercial and residential development increases impervious surfaces that facilitate runoff of a wide variety of pollutants (i.e., vehicle-related chemicals, asphalt leachates, termiticides, landscape pesticides etc).

Coral reefs along the West Maui coastline have been monitored for over 20 years (Williams 2008). Nine reefs monitored in the West Maui area lost significant living coral tissue between 1994 and 2006 (35% to 27% mean coral cover) and an undetermined amount since (Williams 2008). Much of the scientific activity has been focused in the Kā'anapali watershed over the last 5-10 years. Results show increased nutrients and algal blooms (Dailer *et al.* 2010), which prompted studies of wastewater effluent plumes from the Lahaina Waste Reclamation Facility and freshwater seeps on nearshore reefs (Dailer *et al.* 2012) from injection wells. Hunt and Rosa (2009) also reported a multitude of wastewater components from municipal injection wells in Kihei and Lahaina that included personal care products, pharmaceuticals, plasticizers, a fire retardant and musk fragrances. However, to date, no clear causal relationships have been identified that link specific stressors to coral reef impacts which have resulted in direct mitigation or restoration actions.

Other than measures of coral cover, the only other data characterizing the physiological condition of coral along West Maui's coastline was gathered in 2006 (Downs, unpublished data). This cellular physiological data indicated that Honokeana, Kapalua and Honolua Bays had highly elevated biomarker signals in the tissues of the coral *Porites lobata* compared to those at the La Perouse reference site. The bioindicators showed elevated antioxidants, increased oxidative DNA damage, and elevated multidrug resistance enzymes, pointing to localized anthropogenic stressors impacting coral health, but likely differ depending on the specific location. Based on these physiological signals of distress, a survey was undertaken of Kahekili and other embayments along the Maui coastline, as part of this project, to determine locations of concern based on toxic effects of sediment porewaters. In this section, we

provide the toxicity data results from a survey along the Maui coastline, including a concentrated effort in the Kahekili nearshore, which identifies sites exhibiting environments that are toxic to marine life.

II. METHODS

A. Sampling Locations

1. Kahekili Sites

Surface sediments were collected at 7 sites in September 2012 and at 6 sites in June 2013 in the nearshore of Kahekili (Table 9). Collection sites were chosen based on previous research. The overall site selections in 2012 were made with guidance from Wendy Wiltse (U.S. EPA, Honolulu HI) that focused on two features. The first were the Kahekili 'live and dead zones' identified in work by Megan Ross and Paul Jokiel (2010). This work involved mapping Kahekili coral reefs showing 'live and dead zones' with varying degrees and types of degradation, primarily from algal overgrowth that increased in closer proximity to effluent inputs. The second were freshwater seeps that had been identified in work by Meghan Dailer's lab (2010, 2012) that characterized freshwater seeps and measured nutrients and algal blooms in the nearshore of Kahekili helped identify sites with seeps for sampling.

The six sites sampled in 2013 (Table 9) were selected on the advice of Dr. Darla White of the State of Hawai'i's Department of Land and Natural Resources (DLNR), who also conducted the sampling. Dr. White is a coral expert for DLNR and conducts regular monitoring activities in this area. Sites were selected based on knowledge of the freshwater seeps, history of the sites, proximity to 2012 sites, and coral and fish habitats of interest to DLNR.

Table 9. Coordinates for Sediment Porewater Sampling Points near Kahekili.

Region (see map Fig. 12)	September 2012 Sampling Locations	GPS Coordinates		June 2013 Sampling Locations	GPS Coordinates	
1	WNA	20.94098	- 156.69263	Honokowai Point	20.94970	- 156.69195
2	North Seep/CN	20.940192	- 156.69280	-	-	-
	South Seep	20.93850	- 156.69320	Runway	20.93956	- 156.69360
	South of S. Seeps	20.93794	- 156.69357	South Seeps	20.93862	- 156.69315
3	Weston Dead B (WDB)	20.937272	- 156.69374	Bone Yard	20.93733	- 156.69385
	Weston Dead A/ Dead 2 (WDA)	20.937245	- 156.69371	-	-	-
	Weston Live B (WLB)	20.93717	- 156.69338	-	-	-
4	Weston Live A (WLA) /Live 2	20.936444	- 156.69361	Sand Channel	20.93631	- 156.69334
5	-	-	-	Black Rock	20.92951	- 156.69501

'-' indicates that the site was not sampled; CN was water column sample taken at sediment sample site WNA.

2. Other Maui Sampling Sites

Surface sediments were collected at 15 sites in September 2012 and at 10 sites in June 2013 outside the Kahekili area (Table 10; Appendix II) to determine if toxic conditions existed in other embayments along the Maui coast. Sites were selected by the project team based on different types of land-use that carried the potential for varying types of pollutants at each location. The sites included resorts, beach parks, urban centers, areas slated for development and a low-visitation remote reference site (La Perouse). All locations were accessible from shore. Dr. Downs sampled in 2012 and the NOAA team (Charleston SC) sampled in 2013.

Table 10. Coordinates of Other Sediment Porewater Sampling Points along the Maui Coast

September 2012 Other Maui Locations	GPS Coordinates		June 2013 Other Maui Locations	GPS Coordinates	
Honolua Bay	-	-	**Honolua Bay**	21.01373	W 156.63853
Kapalua North	21.000369	- 156.666989	**Kapalua North**	21.00037	- 156.66699
Kapalua South	20.99895	- 156.66757	**Kapalua South**	20.99895	- 156.66757
Napili North	20.996619	- 156.666798	**Napili North**	20.99659	- 156.66672
Napili South	-	-	**Napili South**	20.99464	- 156.66748
Honokeana	20.991592	- 156.668678	**Honokeana**	20.99229	- 156.66890
Kahana Bay	20.985107	- 156.672544	-	-	-
Lahaina	20.888161	- 156.685232	-	-	-
Olowalu North	20.809293	- 156.614236	**Olowalu North**	20.80902	- 156.61353
Olowalu South	20.808366	- 156.605604	**Olowalu South**	20.80911	- 156.61133
Kalaepohaku Pier	20.780806	- 156.463227	-	-	-
Lipoa Place, Kihei	20.745635	- 156.458012	-	-	-
Ulua Beach Park	20.691329	- 156.445376	-	-	-
Wailea, Polo Beach	20.674900	- 156.444396	-	-	-
Makena Beach Park, Little Beach	20.634233	- 156.45198	-	-	-
Makena Rd	20.621762	- 156.439411	**Makena Rd**	20.61763	- 156.41592
La Perouse	20.590683	- 156.412983	**La Perouse**	20.590683	- 156.41298

'-' indicates that the site was not sampled

B. Sediment Porewater Collections

Sediment porewater was collected from 22 sites in 2012 and 16 sites in 2013, along the Maui coastline from Honolua to La Perouse (Tables 9, 10). All sample handlers and their gear were cleaned with Liquinox laboratory detergent and they donned gloves prior to entering the water. Samples were collected within 30 meters of shore in proximity to living coral, even if populations were very small. Lipoa Place (Kihei) and Lahaina in 2012 (Table 10) had no observable living coral; however coral rubble was abundant, indicating former coral habitat. Surface sediment samples were collected using a modified syringe method (Downs *et al.* 2011; training video http://cdhc.noaa.gov/education/field_health.aspx) and transferred into PFA-Teflon bags (Welch Fluorocarbon, Dover NH) and stored frozen until extraction. Porewater was collected from sediment by gravity or vacuum extraction, clarified by centrifugation in Teflon centrifuge tubes (1200 x g for 20 min) and tested for salinity, pH, ammonia, dissolved oxygen (DO) and alkalinity. Samples were amended to ensure that salinity, DO or pH did not confound the results and adjustments were noted to assist in interpreting assay results.

C. Porewater Toxicity Testing

The NOAA NOS Charleston Laboratory (Charleston SC) conducted porewater toxicity testing on Kahekili 2012 and all of the 2013 samples while Haereticus Environmental Laboratory (HEL; Amherst VA) performed toxicity testing on Maui 2012 porewater samples from sites outside Kahekili with sea urchin embryos and coral cells. In addition, HEL also conducted an initial Phase I Toxicity Identification Evaluation (TIE) on select Maui porewater samples that tested positive during porewater toxicity testing.

1. Water Quality Analysis

Salinity was verified for each porewater sample (target 35.0 ±0.5 ppt). Following salinity determination, a 5-mL aliquot was removed to a clean 20 mL glass vial and dissolved oxygen and pH were measured using probes connected to a Thermo Orion 5-Star multimeter. Total ammonia nitrogen (TAN) was determined using a colorimetric based commercial kit (Red Sea, Houston, TX). Kits were modified by creating standards for the assay using 100 mg/L ammonia standard (Hach, Catalog #2406549) in a two-fold dilution series (0.13-8.0 mg/L) in 35 ppt filtered artificial seawater (Sigma Sea Salts: SSS). Un-ionized ammonia (UAN) was calculated from Bower and Bidwell (1978). Following water quality analysis, samples were aliquoted (2 mL, 4 replicates) into new, conditioned (5 mL FASW, 35 ppt), 20-mL glass vials and brought to 25.0 ± 0.5 °C in an environmental chamber prior to the addition of fertilized sea urchin embryos.

2. Sea Urchin Embryo Development Toxicity Assay

Toxicity was determined according to standard methods (Carr and Chapman 1992; Carr *et al.* 1996; Carr and Nipper 2003; ASTM 2012; Appendix III). Sediment porewater toxicity analyses were conducted by NOAA NOS (Charleston SC) and Haereticus Environment Laboratory (HEL, Amherst VA). NOAA assays were conducted with sea urchins (*Arbacia punctulata*) collected off the coast of South Carolina by South Carolina Department of Natural Resources (SCDNR) personnel. HEL assays were conducted with sea urchins (*Lytechinus variegatus*) purchased from Gulf Specimen Marine Laboratory (Panacea, FL). The use of two different species was due to the timing of available gravid urchins. Either species can used in this

bioassay. Gravid sea urchins held at their optimal temperature aquarium systems containing artificial seawater (Aquarium Sea Salt Mixture, Instant Ocean, Blacksburg, VA, 35 ppt). Lighting was provided on a 12h:12h light:dark cycle. Urchins were fed a rotating diet of organic carrots, organic spinach, and seaweed (Julian Sprung's Sea Veggies®) two-three times per week.

Arbacia punctulata sea urchins were spawned by electrical stimulation using a variable voltage transformer. Eggs were collected by inverting the female urchin over a beaker filled to the brim with artificial seawater (35 ppt, 20°C). The urchin aboral side was slightly submerged, so that the eggs were extruded directly into the seawater. After spawning was complete, the eggs were washed three times with an equal volume of fresh artificial seawater (SSS, 35 ppt) and enumerated on a Sedgewick-Rafter counting chamber. Sperm was collected dry by aspiration with a micropipet tip and placed in a sterile 0.5 mL polypropylene Eppendorf tube. Sperm was kept chilled (not directly on ice) until used. Sperm was diluted 1:250 in FASW to activate and cell concentration was determined and motility was verified from a 1:2000 dilution in FASW. Prior to beginning the assay, optimal fertilization rates (>97%) were determined using four dilutions of sperm in a fertilization pre-test. Embryos (~200 in 50 µL volume) were placed in 20 mL glass scintillation vials containing 2 mL of porewater (n=4/sample). Filtered artificial seawater (35 ppt) and 4 mg/L sodium dodecyl sulfate in FASW were included as assay controls. Embryos were incubated for 48 h at 20 ± 0.5 °C under ambient fluorescent lighting on a 12h:12h light:dark cycle. Following incubation, an equal volume of 2X buffered zinc-formalin fixative (Z-fix, Anatech, Poughkeepsie, NY, prepared from concentrate) in FASW (final salinity 35 ppt) was added to each vial. Embryo developmental stage and developmental aberrations were scored using the criteria of Pagano *et al.* (1986), with a target of 100 embryos evaluated per sample replicate.

Lytechinus variegatus required injection of 1-2 mL of 0.5M KCl to initiate spawning. Gametes were collected and fertilization initiated as described above. The *L. variegatus* embryo toxicity assay was conducted in PTFE-Teflon® 24-well microplates. Two milliliters of each porewater sample were placed into 4 replicate wells of a Teflon® 24-well plate. Embryos were added in a 200 µL volume (20-40 embryos) to each well, decreasing the sample concentration to 90%. Sea urchin embryos were incubated at 26°C for 56 h until the control-artificial seawater treatment reached early pluteus stage). Sea urchin plutei were then scored based on deformities, developmental arrest, or mortality (Pagano *et al.* 1986) unfixed.

3. Coral Cell Toxicity Assay

Coral fragments (*Porites divaricata*) were shipped from U.S. NOAA NOS CCEHBR's Coral Culture and Collaborative Research Facility (Charleston, SC; FL Keys National Marine Sanctuary permit FKNMS-2011-161) to HEL where they were grown in a recirculating aquarium with artificial sea water for over four months. This species was selected because the methodologies had been developed for cell isolation and culture for this species and availability. Coral calicoblast cells were isolated using a method described by Downs (2010). Cells were incubated in cell culture media for 8 h and then placed in 250 µL of porewater that had been adjusted for pH, salinity and DO. On average, about 250,000 cells were added to each microplate well. Cells were incubated for 8 h and then scored. Cells were stained with a dye-exclusion viability stain (Naphthol Blue Black) and 10 uL of cell stained suspension was counted with a Neubauer

Improved hemocytometer counting chamber. The resulting live and dead cells were then multiplied by the dilution factor and expressed as a percentage.

D. Phase I Toxicity Identification Evaluation (TIE)

Phase I Toxicity Identification Evaluation (TIE) methods are designed to characterize the toxicity of a sample. U.S. EPA guidelines (U.S. EPA 2007) were used to conduct Phase I TIEs for 7 Maui porewater samples that were found toxic in the initial porewater screening sea urchin development assay. Two solid-phase extraction (SPE) cartridges, HyperSep C18 and Verify-AX (Thermo Fisher, Pittsburg PA), were used separately to fractionate a portion of the original porewater based on its physicochemical properties and associated binding affinity to a particular matrix. This approach helps to determine the general category of the contaminant(s) causing toxicity of the sample. The HyperSep C18 treatment was used for all samples; however, due to sample limitation the Verify-AX was used on only four of the 7 samples. Fractionated porewater eluates from each column were subjected to the sea urchin embryo development toxicity assay described above using *Lytechinus variegatus* and evaluated for toxicity reduction in the sea urchin embryo development bioassay.

E. Phase II Toxicity Identification Evaluation

Phase II TIE procedures (U.S. EPA 2007) are designed to identify possible toxicant(s) implicated in Phase I, thus focusing the analytical chemical analysis more precisely. SPE columns from three samples were selected for preliminary Phase II TIE evaluations. Two were from Kahekili that were seep related (South Seep and South of South Seep) and requested by W. Wiltse (EPA) and a third (Kapalua) that tested positive in the initial porewater test and in the Phase I TIE. Kapalua also had previous data documenting recent degradation of the site. Samples were sent to Jupiter Labs (Jupiter, FL) for contaminant chemistry screening. Kahekili sample, South Seep (SS), porewater was fractionated with SPE columns: Strata AN (Phenomenex, Torrance CA), HyperSep C-18 and Verify AX (Thermo Scientific, Pittsburgh PA). Kahekili sample, South of South Seep (SoSS), porewater and Kapalua North porewaters were fractionated with Bond Elute-C18 (Agilent Technologies, Santa Clara CA) and Verify AX (Thermo Fisher, Pittsburgh PA).

The samples were eluted from the columns and tested for organophosphate pesticides (OP), herbicides and pharmaceutical and personal care products (PPCP), steroidal compounds and estrogenic mimics. For OPs herbicides and PPCPs, the samples were extracted with 5mL of methanol under aggressive vacuum, filtered and then 500 µL was added to mobile phase A (UP H_20 ammonium acetate / formic acid) to insure chromatographic peak shape. For steroidal compounds and estrogenic mimics, the samples were treated under pH conditions to increase mass spectrometry source ionization during the HPLC/MS/MS analysis. All samples were run using the AB SCIEX 5500 Q-Trap with blanks and controls to insure the quality of the data.

F. Statistical Analyses

Results were statistically analyzed using one-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test for treatments versus control. The data were tested for the assumptions of parametric ANOVA, namely that the residuals were found to be normally distributed [Shapiro-Wilks (W)] and homogeneous among groups [Levene's (F)]. Each data set passed or had only very mild departures from these assumptions. All analyses were performed using the SAS statistical package (Version 9.4, SAS Institute, Cary, NC, USA). Alpha was set at 0.05 for all tests. Data are reported as means ± SE.

III. RESULTS & DISCUSSION

A. Sediment Porewater Toxicity Findings for Sites near Kahekili, West Maui

Depending on the particular porewater exposure, a spectrum of morphologies were found in embryos and larvae that ranged from normal plutei larvae to arrested blastula embryos (Fig. 5A-I). Four categories were used to score development: normal (Fig. 5A-B), retarded development (Fig. 5C, E), malformed (Fig. 5D), and arrested development (Fig. 5F-I).

Among the September 2012 samples, three of the 6 porewater samples showed toxicity and were significantly ($p<0.001$) different from the artificial sea water control: SoSS, WDB and WLA (Figs. 6, 7A). The most common abnormality across all three was retarded development displaying under-developed pre-oral arms and smaller in overall size (i.e., dwarfs), similar to Fig. 5C. Embryos incubated in porewater from WDB and WLA also displayed malformed embryos (16% and 29%, respectively, Appendix IV) that were arrested at prisms, absent guts, excessive asymmetry and torsion and similar to those in Fig. 5D-F. Interestingly, one water sample showed enhancement over controls: CN ($p<0.001$).

Among the June 2013 samples, 5 of the 6 samples resulted in 100% toxicity (Fig. 7B; Appendix V; $p<0.001$). Black Rock, the exception, demonstrated 103% normal development compared to the artificial seawater control. Embryos incubated in porewater from Honokowai Point were dead or deformed (Fig. 5D-F) or arrested in larval development (Fig. 5G-I). Embryos incubated in porewater from Runway were predominantly deformed or had arrested development similar to those in Fig. 5F-I. Embryos incubated in the porewater adjacent to the South Seep were mostly dead, unhatched blastula embryos similar to Fig. 5H. Porewater from Bone Yard yielded 100% malformed larvae similar to those in Fig. 5D-F. Embryos incubated in porewater from Sand Channel were primarily dead or arrested at early embryonic development similar to those in Fig. 5G-I. All water quality parameters tested were within acceptable limits with the exception of ammonia. Un-ionized ammonia may have contributed to the toxicities observed. For instance, Black Rock with no detectible toxicity registered 0.1 µg/L, while Runway, the Seep site, and Bone Yard registered 62.9, 78.6 and 64.8 µg/L, respectively. The published NOEC for un-ionized ammonia toxicity is 30 µg/L while the LOEC is 90 µg/L for *Arbacia* embryos (Carr *et al.* 1996). The origin of the ammonia and the role it may play in the toxicity associated with these samples is unknown. It should be noted that toxicity can be ephemeral if the toxic substance is not a persistent contaminant, is delivered in pulses, has a spotty distribution or there is good flushing and turn-over of sediments at a site. This situation was detected at three sites, Bone Yard (BY), Sand Channel (SC) and Runway (RW) after they were re-sampled 5 days after the initial sampling event. When tested, the porewater had significantly lower levels of unionized ammonia, Bone Yard 4.5 µg/L vs 64.8 µg/L, Runway 6.3 µg/L vs 62.9 µg/L and normal development in the bioassay.

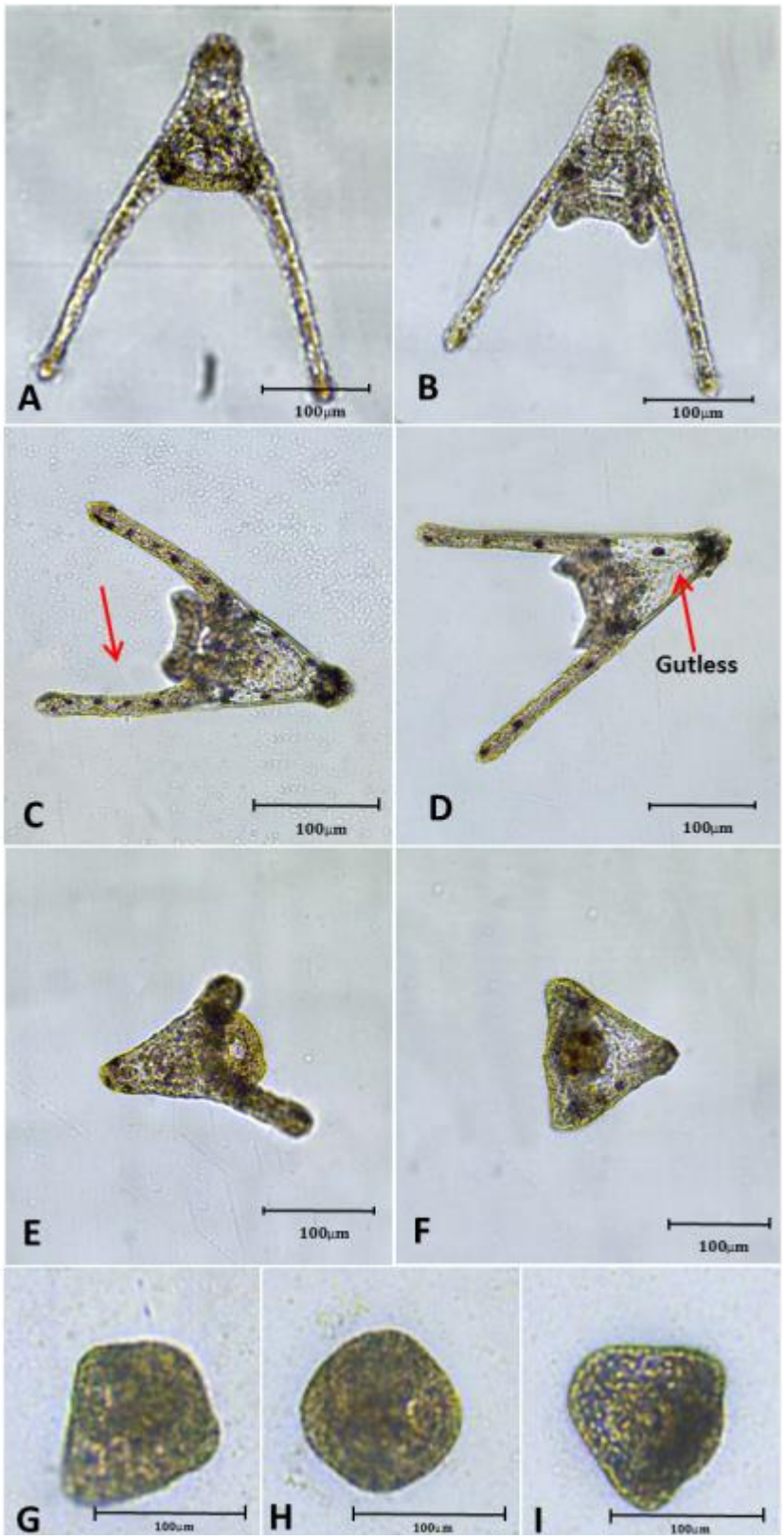

Figure 5. Spectrum of sea urchin (*Arbacia punctulata*) embryo and larvae morphological abnormalities from porewater exposures. A, B=**normal** plutei development; C=**retarded**: under-developed pre-oral arms; D=**malformed**: pathologic plutei, no gut; E=**retarded**: under-developed plutei; F=**arrested** development at prism stage; G-I=**arrested** development: pathologic embryos, blocked at blastula/gastrula.

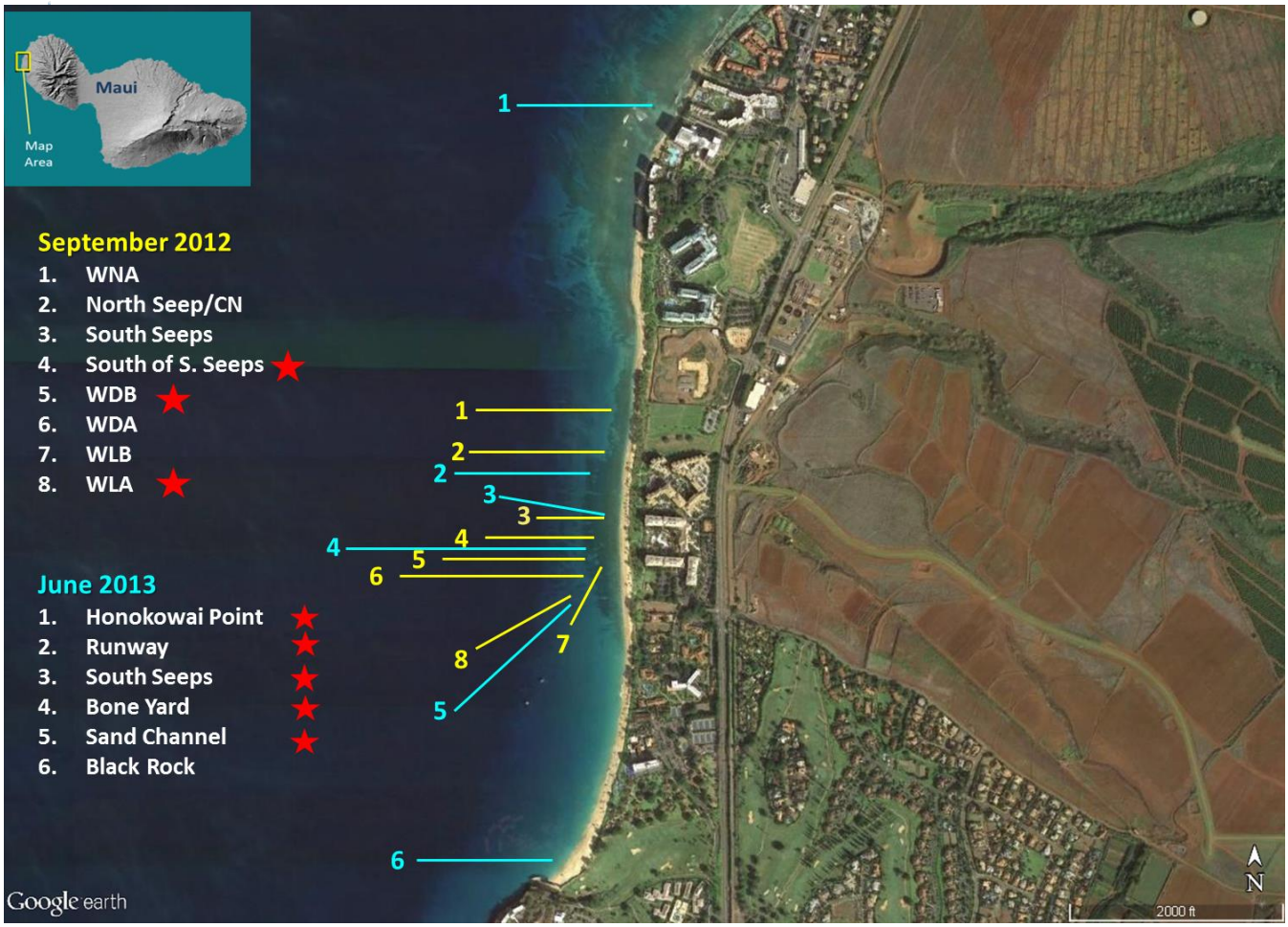

Figure 6. Map of sediment porewater sampling sites along Kahekili nearshore. Sites sampled in 2012 are represented in yellow numbers and lines; 2013 represented in aqua numbers and lines. Red stars=sites testing positive for toxicity in the sea urchin embryo development bioassay. CN was water column sample taken at sediment site WNA.

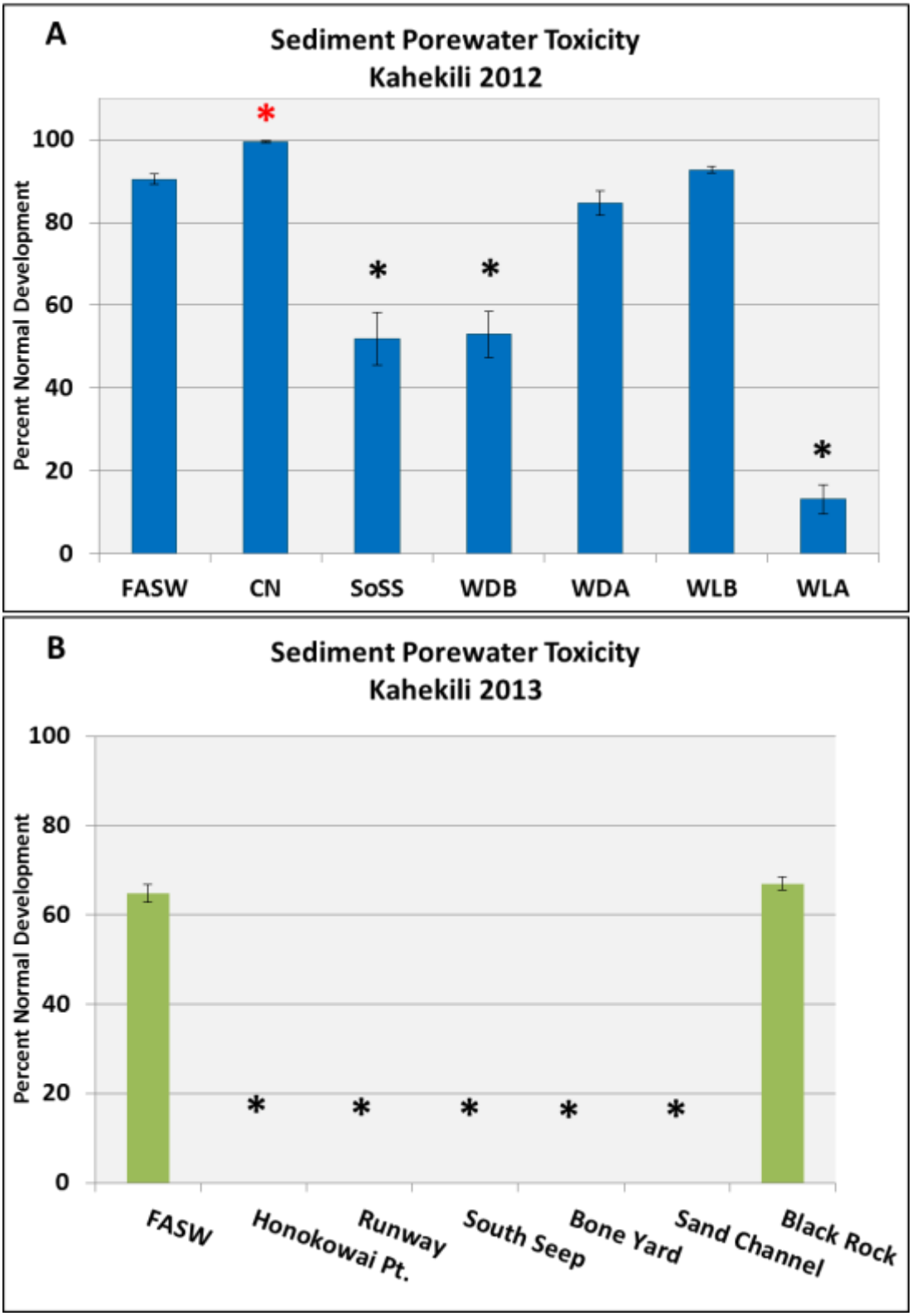

Figure 7. Sea Urchin Porewater Tests: Comparison of mean percent normally developed larvae (± standard error of the mean) of *Arbacia punctulata* exposed to porewater at sampling points along the Kahekili coastline. A=2012; B=2013. Red asterisk=control water column sample showing enhanced development. Treatment means with asterisks significantly differed from the mean ($p<0.001$) based on one-way ANOVA followed by Dunnett's test.

B. **Sediment Porewater Toxicity Findings for Other Sites along the Maui Coastline**

1. **Porewater Toxicity Assays using Sea Urchin Embryos**

In September 2012, 10 of the 15 sites tested positive for toxicity and were significantly different from the reference site, La Perouse ($p<0.001$; Fig. 9A; Appendix VI). No normal plutei were present in treatment porewaters from Kapalua Bay South, Honokeana, Lahaina or the Pier at Kalaepohaku (in Kihei). Samples displaying the highest mortality were from Lahaina (100%) and Honokeana Bay (49.65%) (Fig. 9A; Appendix VI) with embryo morphologies similar to those in Fig. 5G-I. Honokeana also displayed marked toxicity with deformed plutei (50.35%) along with samples from the north side of Kapalua Bay (68.04%) (Fig. 9A; Appendix VI). Samples from the south side of Kapalua Bay showed a different pattern with 46.07% arrested in development similar to Fig. 5F-I, 35.3% deformed and similar levels of mortality with samples from the north side of the bay. Samples from the Pier at Kalaepohaku, however, showed the highest level of developmental arrest (73.38%) among all sites tested (Fig. 9A; Appendix VI). Three samples tested from Kahekili showed less than 10% of the embryos displaying any sign of toxicity. The highest among this particular location was a site near the WNA with 10.2% deformed embryos and 8.5% mortality (Appendix VI).

In June 2013, only one of the 6 samples, south side of Kapalua Bay, tested positive for toxicity and was significantly different from the artificial seawater and reference site (La Perouse) controls ($p<0.001$) (Fig. 9B; Appendix VII). Embryos treated with porewater from Kapalua Bay South displayed 35.8% retarded development similar to Fig. 5C, E and 27% arrested embryos similar to Fig. 5F-I.

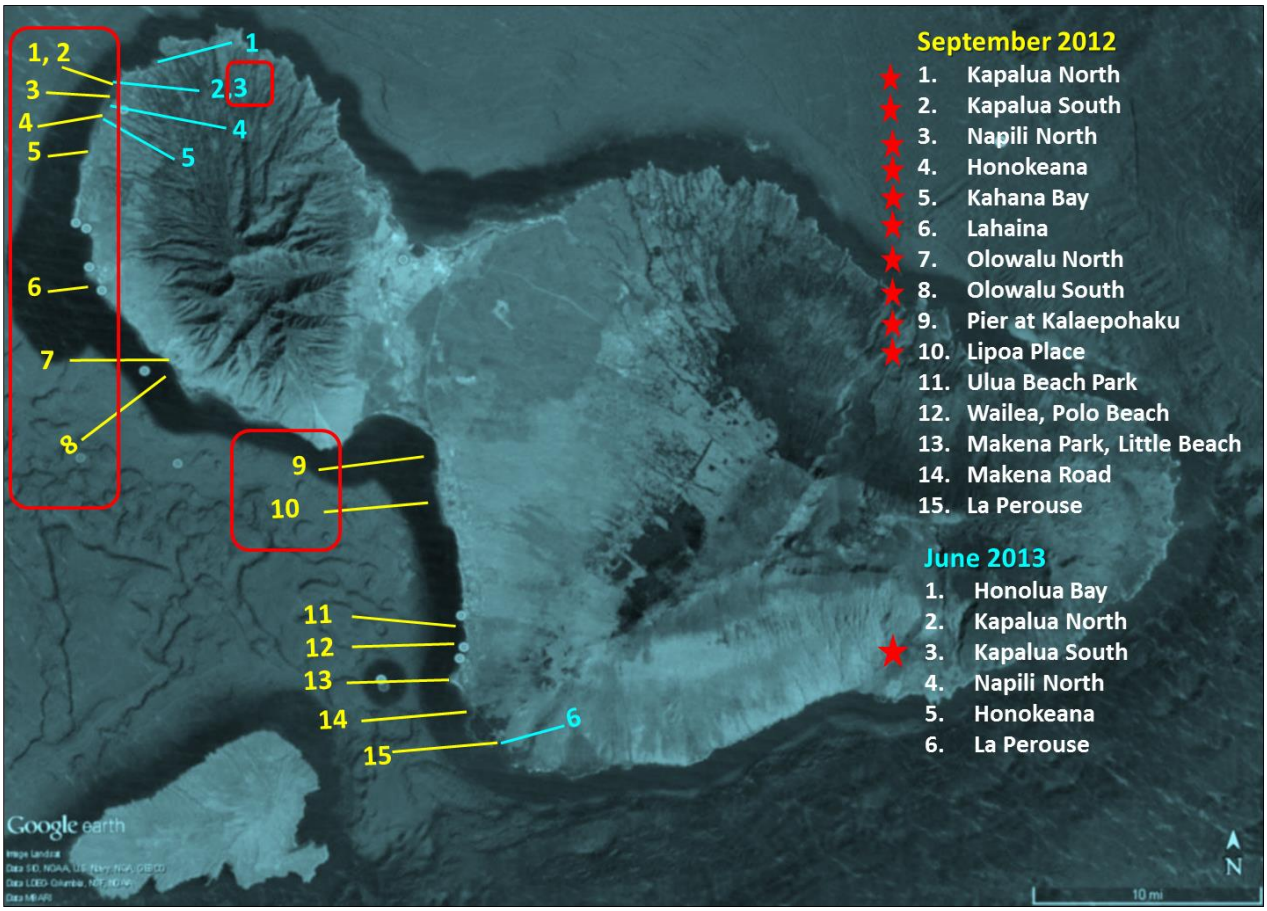

Figure 8. Map of sediment porewater sampling in 2012 (yellow) and 2013 (blue) at points along the Maui coastline. Red stars and boxes = sites testing positive for toxicity with the sea urchin embryo development bioassay.

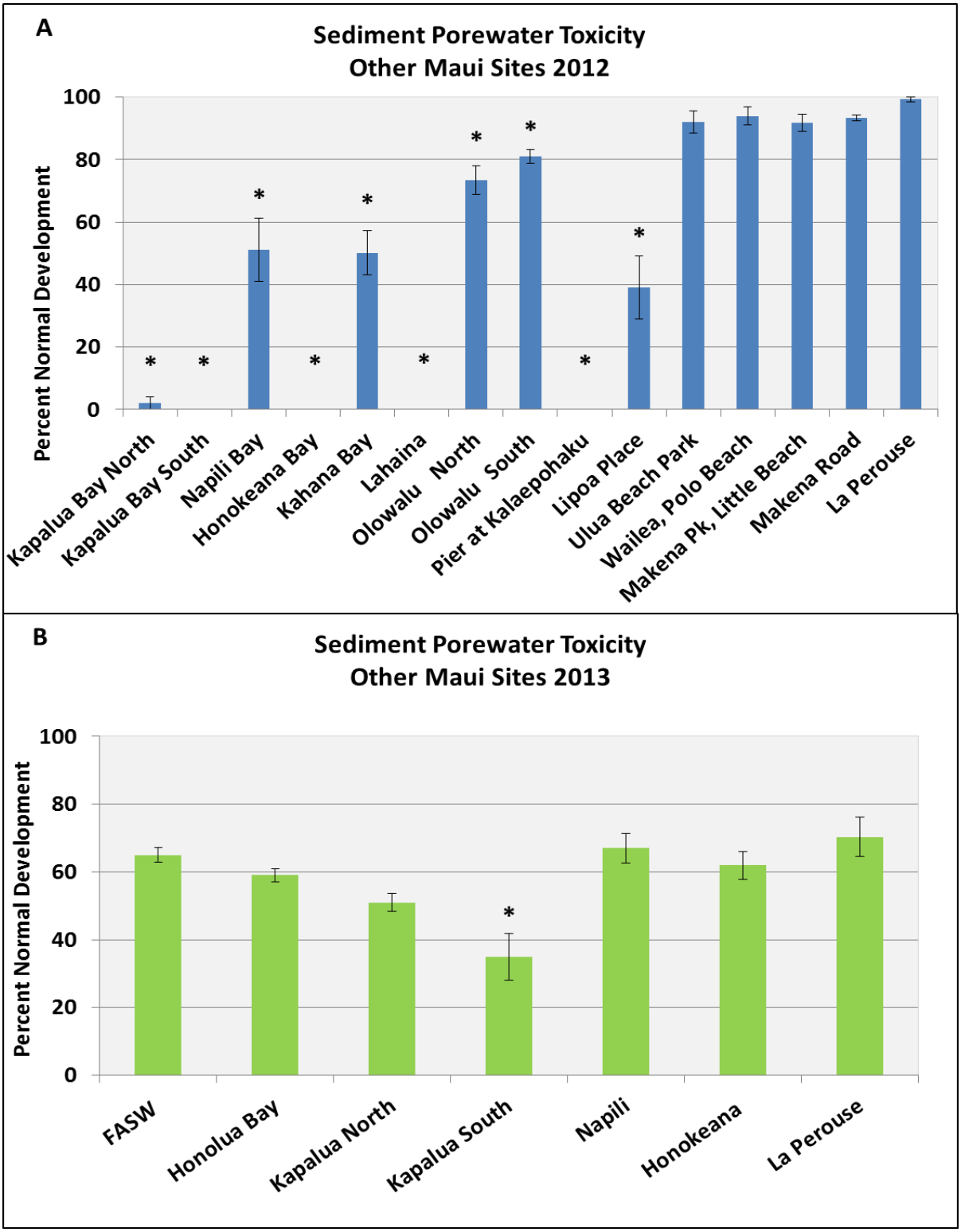

Figure 9. Sea Urchin Porewater Tests. Comparison of mean percent normally developed larvae (± standard error of the mean) at sampling points along the Maui coastline. A=2012 *Lytechinus variegatus* used for bioassay, La Perouse used as reference control; B=2013 *Arbacia punctulata* used for bioassay, FASW used as control. *=Treatment mean differed from the control $p<0.001$ based on one-way ANOVA followed by Dunnett's test.

2. Porewater Toxicity Assays using Coral Calicoblast Cells from *Porites divaricata*.

Based on the findings from the sea urchin toxicity test, four porewater samples from 2012, exhibiting toxicity were used in a coral cell toxicity assay. Primary cell cultures of *Porites divaricata* calicoblasts were exposed to porewater from north Kapalua Bay, south Kapalua Bay, Pier at Kalaepohaku, Lipoa Place and La Perouse (reference site) (Fig. 10). As shown in Figs. 9A and 10, each of the sites toxic to sea urchin embryo development were also toxic to coral calicoblast cells. Three sites (south Kapalua, Pier at Kalaepohaku, Lipoa Place) were significantly different from ASW and reference site controls at ($p<0.001$). North Kapalua Bay was significantly different from controls at $p<0.04$. La Perouse, a long-term reference site was not different from the artificial seawater control.

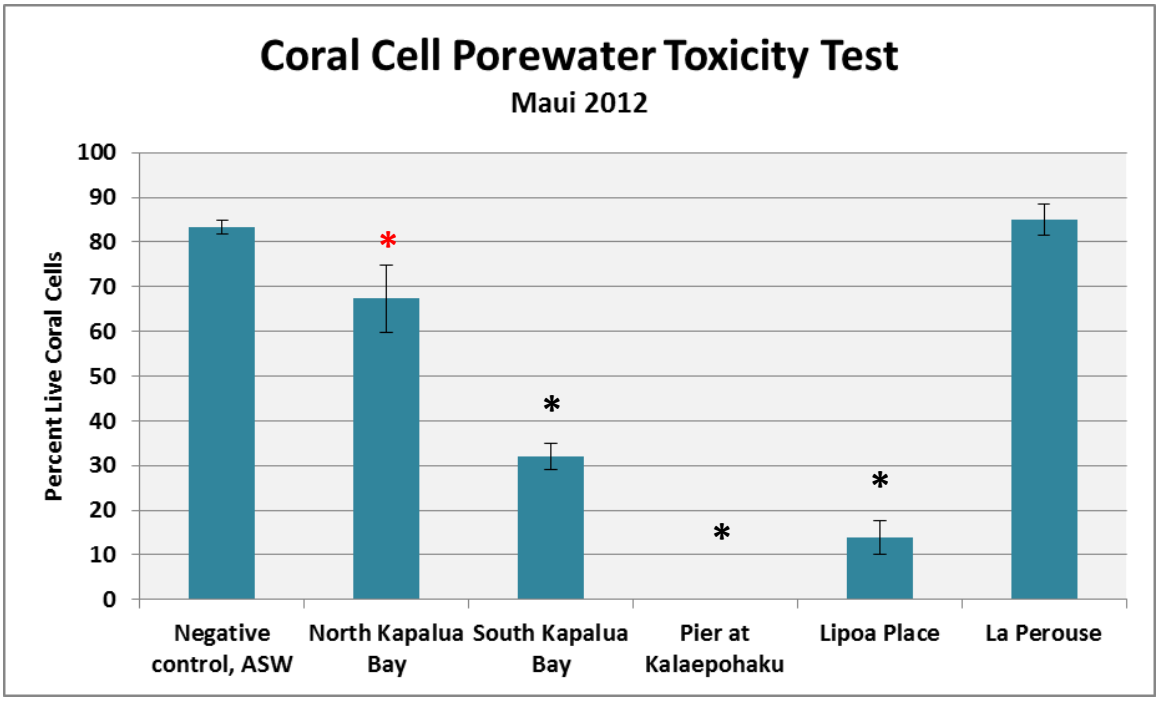

Figure 10. Coral Cell Porewater Toxicity Test using Calicoblast Cells. Porewater from 5 of the sea urchin tested samples (Fig. 9A) were tested for toxicity against *Porites divaricata* calicoblast cells. Toxicity is expressed as percent live cells remaining at the end of the exposure for the assay endpoint. Treatment mean with red asterisk differed from the mean $p<0.04$, black asterisk=$p<0.001$ based on one-way ANOVA followed by Dunnett's test.

3. Phase I Toxicity Identification Evaluation

Based on the porewater findings with sea urchin embryo development in 2012, and the availability of porewater, a Phase I Toxicity Identification Evaluation (TIE) was conducted. This included samples from Kapalua Bay north, Honokeana, Kahekili North of Plume, Olowalu north, Olowalu south, Pier at Kalaepohaku and Lipoa Place (Fig. 8). Note that Olowalu samples were sufficient for only one replicate each.

Porewater from these sites was treated with two types of solid phase extraction (SPE) columns to determine the general category of the pollutant that is causing toxicity. Because of a lack of ample porewater, not all toxicity reduction treatments could be conducted. A HyperSep-C18 SPE treatment was used for all samples. These columns are broad spectrum binding to nonpolar to moderately polar compounds (e.g., organics) such as pharmaceuticals, THC, organochlorine insecticides, pesticides, PAHs and explosives (Thermo Scientific 2007). Four samples (Kapalua Bay north, Kahekili North of Plume, Pier at Kalaepohaku and Lipoa Place) had sufficient porewater to treat with a second column, the HyperSep Verify-AX SPE cartridge. This column is based on two functional groups: reversed phase C8 and a quaternary amine anion exchanger which binds moderately polar to non-polar and ionized and charged compounds. It is commonly used to bind acidic drugs and their metabolites, glyphosate and glufosinate and metals (Thermo Scientific 2007).

Once porewater was treated with these different columns, the eluent was used in the sea urchin embryo development bioassay. Sea urchin embryos were incubated for 56 h (early stage pluteus), and scored for mortality, arrested development or deformities.

Each treatment removed some amount of toxicity from each sample (Fig. 11; Appendix VIII), though only at Kahekili WNA and Olowalu north was all toxicity removed, although toxicity was not high in the original sample (approx. 20-25%, respectively). The most dramatic reductions in toxicity were with the C18 column SPE treatment which removed most of the toxicity associated with samples from the Pier at Kalaepohaku (0% normal embryos before SPE to 90% normal embryos after SPE). Though not quite as dramatic, toxicity was decreased in the sample from Honokeana Bay (0% normal embryos to 77% normal embryos). Similar reductions in toxicity with the C18 SPEs also were observed at Kapalua Bay North (2% normal embryos to 83.2% normal embryos). In contrast the toxicity present in the Lipoa Place porewater was not significantly reduced by the HyperSep C-18 column treatment. However when treated with the HyperSep Verify AX column, toxicity from the Lipoa Place sample was completely removed, indicating the possibility of metals or broad spectrum herbicides such as glyphosate (e.g., Round-up™) or glyfosinate (e.g., Ignite™) used for weed-killing. The HyperSep Verify AX treatment of the porewater from the Pier at Kalaepohaku performed similar to the HyperSep C-18 treatment by removing most of the toxicity and increasing normal development from 0-90%. The Verify AX treatment was marginally effective in removing toxicants from Kapalua Bay north and the WNA site at Kahekili. There was not enough porewater remaining from the other sites for this treatment. There was no toxicity associated with the artificial seawater control for sea urchin embryos.

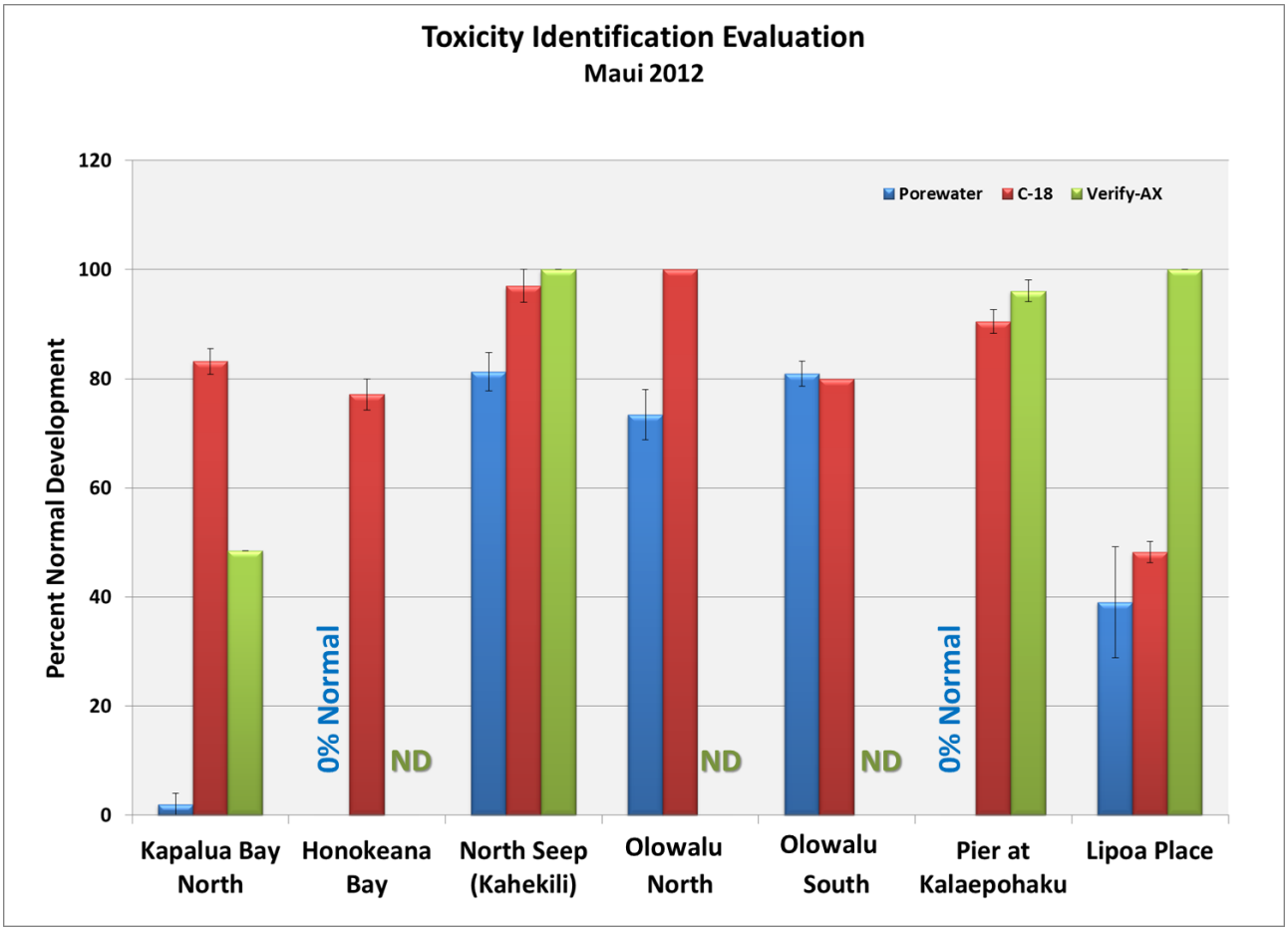

Figure 11. Phase I TIE of select sites along the Maui coastline. Porewater from 7 Maui sites was fractionated with either Hypersep C18 or Verify AX SPE column to remove potential toxicants and eluents were tested in a sea urchin embryo development bioassay using *Lytechinus variegatus* embryos. ND=not determined with Verify-AX. *Note due to limited sample volume only one replicate was possible for the Olowalu samples.*

4. Preliminary Toxicity Identification Evaluation, Phase II

Based on the toxicity reductions identified during the Phase I TIE of samples from 2012, the TIE SPE column from three West Maui sites underwent contaminant chemistry analysis (Appendix IX). In addition to the HyperSep C-18 and Verify AX SPE columns, water from Kahekili South Seep (SS) (which is seep water in proximity to the South of the South Seep) also underwent treatment with a Strata AN SPE column (a precursor to Strata X SPE columns) which binds neutral, acidic or basic compounds and used to extract polar compounds from environmental samples, such as pharmaceuticals and personal care products (PCPs) (Fig. 6). These analyses were done in a screening mode, to identify candidate toxicants at a given location; the analyses are not quantitative. The summary results of these screening analyses are shown in Table 11, instrument level of detection was approximately 5 µg/L (parts per billion (ppb)). Carbamazepine (CBZ) found in both Kahekili porewater samples. Carbamazepine is an antiepileptic, mood stabilizing drug. No studies were found that address the effects of CBZ on coral or other reef organisms. Caffeine, another common analyte found in wastewater and often used as an anthropogenic marker was detected in the South Seep sample. Sawyer and Muscatine (2001) have reported that caffeine can alter intracellular levels of protein phosphorylation and evoke bleaching in laboratory experiments at millimolar concentrations (i.e., parts per million range).

Herbicides were also detected in each of the three samples. Atrazine (triazine herbicide) was detected in the SS sample. Kapalua samples presented with malathion (organophosphate insecticide) and atrazine. Atrazine and simazine (both triazine herbicides) were detected in the South of South Seep porewater (Table 11; Appendix IX). Black Rock Spring, Ka'anapali was the closest site sampled in the 2013-2014 Statewide Pesticide Sampling Pilot Project (HERR 2014) to those of this project. This study detected atrazine, simazine, diuron and iprodione (fungicide) assumed to be inputs from golf courses, urban landscaping, and historic pineapple and sugar cane fields. Atrazine has also been detected in groundwater and wells on Maui. Of these herbicides, bio-effects data for coral are only available for atrazine and diuron (Jones *et al.* 2003). The No Observable Effects Concentration (NOEC) levels for atrazine were determined as 1 µg/L and Lowest Observable Effect Concentration (LOEC) 3 µg/L for photosynthetic efficiency endpoints. Using change in the effective quantum yield of photosynthetic efficiency as an effect criterion, diuron (NOEC = 0.3 µg/L, 300 pptrillion; LOEC = 1 µg/L, 1 ppb; median effective concentration, EC50 = 4 to 6 µg/L) was found to be more toxic than atrazine (NOEC = 1 µg/L, LOEC = 3 µg/L, EC50 = 40 to 90 µg/L) in short-term (10 h) toxicity tests.

Table 11. Summary of Chemical Findings for 3 West Maui Sites.

Analyte	South Seep Strata AN	South Seep HyperSep C18	South Seep Verify AX	Kapalua Verify AX	Kapalua Bond Elute C18	South of South Seep Bond Elute C18	South of South Seep Verify AX
Caffeine	X						
Carbamazepine	X	X	X			X	X
Primidone	X	X	X				
Sulfamethoxazole	X	X	X				
PCF					X		
1-bromo 2 nitro benzene			X				
Atrazine		X			X	X	
Malathion					X		
Simazine						X	

IV. Summary of Toxicity Testing

- Table 12 summary all of the toxicity testing conducted during the project per site.

Table 12. Summary of Toxicity Test Conducted.

	Sea Urchin Porewater Tox.	Coral Cell Porewater Tox.	Phase I TIE	Phase II TIE
Kahekili 2012				
WNA /CN	✓		✓	
South Seeps				✓
South of S. Seep	✓			✓
Weston Dead B (WDB)	✓			
Weston Dead A/ Dead 2	✓			
Weston Live B (WLB)	✓			
Weston Live A (WLA) /Live 2	✓			
Other Maui Sites 2012				
Kapalua North	✓	✓	✓	✓
Kapalua South	✓	✓		
Napili North	✓			
Honokeana	✓		✓	
Kahana Bay	✓			
Lahaina	✓			
Olowalu North	✓		✓	
Olowalu South	✓		✓	
Kalaepohaku Pier	✓	✓	✓	
Lipoa Place, Kihei	✓	✓	✓	
Ulua Beach Park	✓			
Wailea, Polo Beach	✓			
Makena Beach Park, Little	✓			
Makena Rd	✓			
La Perouse	✓	✓		

Table 12 con't. Summary of Toxicity Test Conducted.

	Sea Urchin Porewater Tox.	Coral Cell Porewater Tox.	Phase I TIE	Phase II TIE
Kahekili 2013				
Honokowai Point	✓			
Runway	✓			
South Seeps	✓			
Bone Yard	✓			
Sand Channel	✓			
Black Rock	✓			
Other Maui Sites 2013				
Honolua Bay	✓			
Kapalua North	✓			
Kapalua South	✓			
Napili North	✓			
Napili South	✓			
Honokeana	✓			
Olowalu North	✓			
Olowalu South	✓			
Makena Rd	✓			
La Perouse	✓			

Kahekili

- Of the 12 Kahekili sites analyzed using the sea urchin embryo development assay, one site (CN, 2012 sample set) showed significantly enhanced ($p<0.001$) embryo development as compared the artificial seawater control. This could be due to low levels of nutrients, or natural constituents in the seawater that are not found in artificial seawater mixtures.
- Three Kahekili sites were not significantly different from the control in the sea urchin toxicity assay: WDA and WLB (2012) and Black Rock (2013).
- In 2012, three sites tested positive for toxicity using the sea urchin embryo development assay (SoS, WDB, WLA).
- In 2013, 5 of 6 sites resulted in 100% toxicity with all embryos developing abnormally but with differing abnormalities. Honokowai Point predominantly dead or deformed embryos; Runway predominantly deformed or embryos arrested in development, South Seep had predominantly dead embryos; Boneyard had 100% malformed larvae; and Sand Channel had predominantly dead or embryos arrested early in development.

- Un-ionized ammonia is a suspected candidate contributing to the observed toxicity in the 5 Kahekili samples from 2013. Black Rock with no detectible toxicity registered 0.1 µg/L, while Runway, the Seep site, and Bone Yard registered 62.9, 78.6 and 64.8 µg/L, respectively. The origin of the ammonia and whether it is the only toxicant in the samples are unknown. It should be noted that toxicity can be episodic, particularly if there is significant flushing of an area. This situation was detected at three sites, Bone Yard (BY), Sand Channel (SC) and Runway (RW) after they were re-sampled 5 days after the initial sampling event that showed 100% toxicity. When tested, the porewater had much lower levels of un-ionized ammonia, Bone Yard 4.5 µg/L vs 64.8 µg/L, and Runway 6.3 µg/L vs 62.9 µg/L and the samples tested negative for toxicity.
- Phase I TIE treatment with Verify AX removed 100% of the toxicity in the Kahekili WNA sample. The possible toxicant classes include moderately polar to non-polar and ionized and charged compounds. This treatment is commonly used to bind acidic drugs and their metabolites, glyphosate and glufosinate and metals (Thermo Scientific 2007).
- Contaminant analysis of two Kahekili samples resulted in detection of caffeine, carbamazepine, primidone, sulfamethoxazole, 1-bromo-2-nitrobenzene and atrazine in the South Seep water sample. Carbamazepine, atrazine and simazine were found in porewaters from South of the South Seep samples.

Other Maui Sites

- Of the 25 samples taken outside the Kahekili area and analyzed using the sea urchin embryo development assay, 14 samples were not significantly different from the control: Ulua Beach Park, Wailea Polo Beach, Makena Park, Little Beach, Makena Road and La Perouse (2012) and Honolua Bay, Kapalua north, Napili north, Napili south, Honokeana, Olowalu north, Olowalu south, Makena Rd and La Perouse (2013).
- In 2012, 10 of 15 sites tested positive for toxicity in the sea urchin development assay when compared to the reference site, La Perouse: Kapalua Bay north and south sides of the bay, Napili, Honokeana Bay, Kahana Bay Lahaina, Olowalu north and south sides of the bay, the Pier at Kalaepohaku and Lipoa Place. Only one of six 2013 samples tested positive for toxicity: Kapalua Bay South.
- Four of the five 2012 porewaters tested positive in a coral cell mortality assay: North Kapalua Bay, north and south sides of the bay, the Pier at Kalaepoku and Lipoa Place. La Perouse, the reference site was not significantly different from the artificial sea water control. The difference in toxicity between the north and south side of Kapalua Bay indicates differential pollutant inputs.
- Phase I TIEs of a subset of 2012 samples showed reduction in toxicity in all of the tested samples using C-18 and Verify-AX SPE column treatments. The C-18 SPE treatment

reduced toxicity 100% in the sample from Olowalu South, while reducing the toxicity by ~80% in the Kapalua Bay North sample, by 77% in the Honokeana Bay sample and by 90% in the sample from the Pier at Kalaepohaku. Olowalu's 20% toxicity was not reduced by the C-18 treatment. The Verify AX treatment reduced 100% of the toxicity in the Lipoa Place sample, by 96% in the Pier at Kalaepohaku sample and only 45% in the Kapalua Bay North sample.

- Pollutants in the porewater are predominantly associated with organic derived pollutants (e.g., pharmaceuticals, organochlorine insecticides, pesticides PAHs) as indicated by the TIE (C-18 SPE treatment). Lipoa Place and the Pier at Kalaepohaku toxicity still remained after removing the organic-based toxicants (C-18 treatment). The greater reduction in toxicity by the Verify AX SPE treatment suggests the additional toxicity associated with these two sites could be associated with metals due to its anion exchange properties in addition to binding non-polar and moderately polar organic compounds.
- Results of contaminant analysis of C-18 SPE column used with Kapalua Bay samples indicated the presence of atrazine and Malathion.
- The solid phase columns used in this study, or another similar study, could be used to identify the toxicant directly.

V. Conclusions and Recommendations

1. Results of the toxicity testing and preliminary analytical chemistry indicate pollutants are present in the sediment porewater at multiple locations within the nearshore of Kahekili (Fig. 6). The source of toxicity is unknown but evidence exists from this project and others for groundwater contamination with agrochemicals such as atrazine, as well as contributions from treated wastewater as indicated by the presence of pharmaceutical drugs and caffeine, a common indicator for wastewater, in seep water samples (Table 11).

2. Multiple bays along the West Maui coast (Fig. 8) also tested positive for toxic effects in a sea urchin bioassay. These include Kapalua, Napili, Honokeana, Lahaina, Olowalu, the Pier at Kalaepohaku and Lipoa Place. Toxicity reduction assays suggested site specific sources of toxicity that included herbicides (atrazine and simazine) used in lawn care, metals, polar organic products such as pharmaceuticals and personal care products.

3. The data from this project provides a baseline screen of multiple locations within the Kahekili near shore and other embayments along the Maui coastline for conditions that were shown toxic to marine life. Evidence was provided for a temporal component (i.e., 2012 and 2013 sample analyses) to the presence of

pollutants in the water or sediment porewaters. These locations warrant further investigation to determine the identity of the pollutant, its source and potential control mechanisms.

4. Our data suggest that each location is likely to have a suite of different stressors and/or pollutants. Future studies are needed to understand the underlying drivers causing failure of these individual ecosystems and will require individualized investigations to determine the identity and quantity of key pollutants (e.g., using analytical chemistry) as well as temporal and spatial dynamics of pollutant inputs. This information will help determine the risk potential and mitigation actions necessary to recovery each of the individual systems.

5. Identification of priority pollutants will allow laboratory studies that can determine thresholds at which adverse effects occur for marine life as well as determine additive or synergistic compounds or environmental conditions (e.g., temperature, pH, salinity) that can change threshold concentrations.

6. This work provides a basis for managers to explore technologies to improve the treatment of sewage particularly for pollutants found harmful to marine life, seek opportunities to improve lawn care practices that reduce inputs of fertilizers and herbicides/pesticides used in commercial, agricultural and residential settings.

7. Our results show that pollutants are present in once vibrant embayment along the West Maui coast. Pollutants associated with wastewaters as well as those contained in many personal care products used by beach-goers, for example, contain endocrine disrupting chemicals. These chemicals often cause sublethal effects, i.e., not causing immediate death of marine life. This group of chemicals target reproductive and developmental stages of these organisms. The ability to sexually reproduce is critical for ecosystems and populations to survive and function. Future studies should evaluate the reproductive potential of organisms foundational to the recovery and functioning of these vulnerable systems of West Maui.

8. The watersheds and associated nearshore environments of West Maui have been recognized as a priority site by NOAA's Coral Reef Conservation Program needing effective management strategies to protect and conserve the marine resources. A recommended approach to meet this goal is to investigate the condition and identify impacts; mitigate the sources of impacts to allow recovery and restoration of the important marine resources.

CLASSROOM & LABORATORY TRAINING

OBJECTIVE 3. PROVIDE PRACTICAL TRAINING AND TECHNOLOGY TRANSFER FOR THREAT ASSESSMENTS

I. Background

The effect of anthropogenic activities and pollutants on reef health and vitality is an area of major concern. A threat (or risk) assessment is a tool that can help generate scientific information to use in justifying and determining appropriate management action(s). This method can be a powerful tool in helping determine the probability of a risk to the resource by a particular activity or pollutant, especially when funds, expertise and time are limited. We used this approach to investigate risk factors affecting coral reef degradation in West Maui, Hawai'i.

To aid resource managers in prioritizing potential impacts from human activities, and generate their own data to justify management actions for protecting the resource, the Coral Disease and Health Consortium (CDHC) provided training September 25-27, 2012, in: ***Practical Methods for Conducting Threat Assessments for Reef Managers.*** Hosted by Humpback Whale National Marine Sanctuary in Kihei, Maui, Hawai'i, 18 participants representing local NGOs, and state and federal resource managers were introduced to the concepts of a risk assessment and provided an opportunity for hands-on practical application of the concepts using a local case study at sites within the Kahekili Beach Park, West Maui Hawai'i.

II. OVERVIEW OF AN ECOLOGICAL RISK ASSESSMENT

An Ecological Risk Assessment (ERA) is a process for organizing and analyzing data, assumptions and uncertainties to evaluate the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors (EPA 1998, 1999). This process applies to chemical (pollutant), physical (e.g., habitat destruction) or biological (e.g., introduced species) stressors and is helpful in making informed and scientifically defensible decisions. There are five distinct elements of an ERA: planning, problem formulation, exposure analysis, effects analysis and risk characterization (Fig 12).

A. Planning

The planning process identifies management goals and articulates the characteristics of the ecological values that management wants to protect. Goals that explicitly define ecological values to be protected are the best type of management goals and are the easier ones to determine metrics of success. For example, "Re-establish and maintain water quality conditions to support growth and reproduction of coral and other reef organisms in the West Maui watershed" is good but needs to be further defined with specific objectives about what must occur to achieve the goal and ecological values that are measurable.

B. Problem Formulation

Problem formulation is a process used to generate and evaluate preliminary hypotheses about why ecological effects have occurred or may occur from human activities. As part of the problem

formulation, assessment endpoints are identified that describe the entity to be protected and what the undesirable effects to be avoided are. Examples could include coral reef community structure, coral survival, growth and reproduction or the distribution of critical habitat. Conceptual models are a hallmark of the *Problem Formulation* phase of an ERA. Conceptual models lay out anticipated exposure route scenarios from a source to the receptor of concern. In this case coral is the receptor of concern and exposure routes may include for example sewage seeps, runoff, and/or agrichemical application. The final stage of the problem formulation is an analysis plan to provide a road map for addressing the problem, similar to an experimental design. It seeks to identify as many possible and probable outcomes and their consequences.

C. Exposure Characterization

Exposure characterization is used to estimate or measure the magnitude, frequency and duration of an exposure to a putative stressor that is identified during the problem formulation phase. This element ideally describes the source(s), distribution in the environment and contact or co-occurrence of stressors with the ecological receptors of concern and includes the uncertainties associated with each one. This step usually involves contaminant chemistry analysis or direct evidence of exposure to a given stressor.

D. Effects Characterization

Effects characterization examines the relationship between stressor levels and ecological effects. These relationships may be acute toxicity values, dose-response information or chronic or sub-acute toxicity values. Effects characterization also examines the plausibility that effects may occur or are occurring as a result of exposure to particular stressors and it also provides scientific evidence that exposure to a given stressor causes the observed effects. This requires measureable ecological effects and relevant assessment endpoints. For coral reefs this may involve diagnostic biomarkers or ecological measures such as fecundity and recruitment.

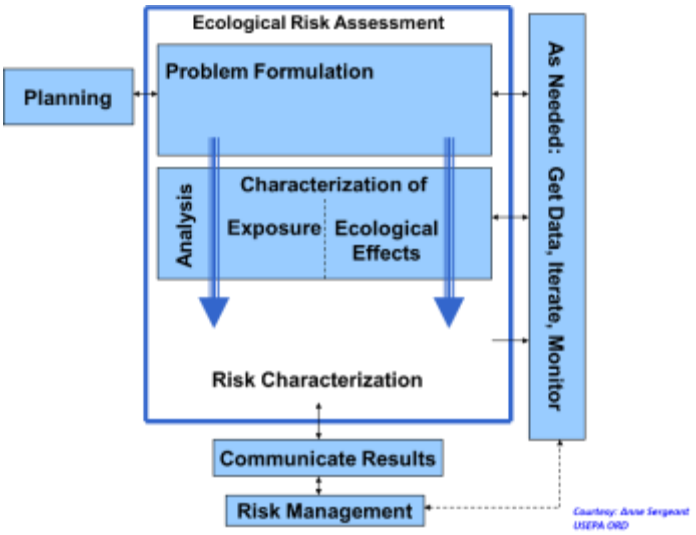

Figure 12. Ecological Risk Assessment Process. The five elements of ERA are shown here: planning, problem formulation, exposure analysis, ecological effects and risk characterization.

E. Risk Characterization

The risk characterization phase of an ERA integrates the stressor-response and exposure assessments, which may involve qualitative as well as quantitative information. A risk model is developed that also describes the uncertainty of the data.

III. LABORATORY & FIELD TRAINING

The laboratory component of the training provided hands-on experience in techniques for 1) bacterial water quality and 2) sediment porewater toxicity testing using the sea urchin development bioassay. Key details of each technique are provided here with detailed protocols available in Appendices I, II, and III.

A. Laboratory Safety Training

Class participants were introduced to potential laboratory hazards including harmful biota, toxicants and select laboratory supplies and chemicals. Students were instructed in the use of various forms of personal protective equipment and when to use it. General laboratory safety guidelines were communicated, including methods for cleaning laboratory areas following work with microbes or toxicants, and procedures to follow for accidental exposures to hazardous chemicals or biota. All participants were trained in the proper use and/or disposal of needles, broken glass, flammable material, and cultured pathogenic bacteria. All class laboratory experiments were conducted without incident.

B. Bacterial Water Quality Method

Bacterial water quality analysis is used to estimate the numbers (or concentration) of certain bacteria of interest in a water sample. Routine screening of water quality uses indicator species of bacteria commonly found in animal feces to show the possible presence of sewage in the water and signal a risk of pathogen exposure. There are a number of methods used in water quality testing. The Membrane Filtration Method was presented in this training class.

1. Water Collection for Bacterial Analysis

Water is collected from target sites into 1 L sterile polypropylene bottles (Fig 13). Bottles can be sterilized using an autoclave or by treating with 70% isopropyl alcohol. We demonstrated using isopropyl alcohol because it is readily available in grocery and drug stores. After rinsing with isopropyl alcohol, a small amount (3-5 mL) is left in the bottle prior to collection. At the sampling site, the sample collector will don nitrile gloves and enter the water to the pre-determined distance and depth from shore (Fig 13). With the sample collector facing 'upstream' or 'against the current' the collection bottle is opened in front and away from the collector. Residual alcohol is first thoroughly rinsed from the sampling

Figure 13. Student conducting water sampling for bacterial water quality testing.

bottles by filling the bottles with sea water and emptying the bottle behind the collector (downstream) three times. The sample is then taken upstream from the sample collector. The seawater is placed at ambient temperature and transported to the laboratory.

2. Membrane Filtration Method for Bacterial Water Quality Enumeration

The Membrane Filtration Method is based on a plate count method which involves vacuum filtering dilutions of the water sample onto gridded nitrocellulose membrane filters that are then placed onto selective agar plates for incubation, allowing bacterial colony growth.

Using a vacuum filtering manifold and sterile magnetic filter funnels (47mm and 300-500 mL capacity), glass fiber filters were first placed onto the filter holder as a support for the nitrocellulose filters and wetted. Nitrocellulose filters were then placed on top of the glass fiber filters and the funnel secured. Samples of 100 mL volume and their dilutions were filtered onto the nitrocellulose filters, each neat and dilution filtered in triplicate. The filters were removed from the holder and placed onto Petri plates containing selective agar media (Fig. 14), then incubated at the prescribed temperature and time for the given media. Selected bacteria present in the water sample will grow into colonies on the filter paper. Bacterial colonies (representing an originating bacterium) were enumerated (Fig. 15) on each plate. Counts from replicate plates were averaged and graphed by the students. For *Enterococcus* which is selected on mEI media (Fig. 15A) the resulting colony forming units (cfu) were compared to state and national water quality standards. Samples were considered positive if they exceeded the single sample limit of 104 cfu/100 mL or potentially positive if there were 35 or more cfu/100 mL of *Enterococcus* enumerated.

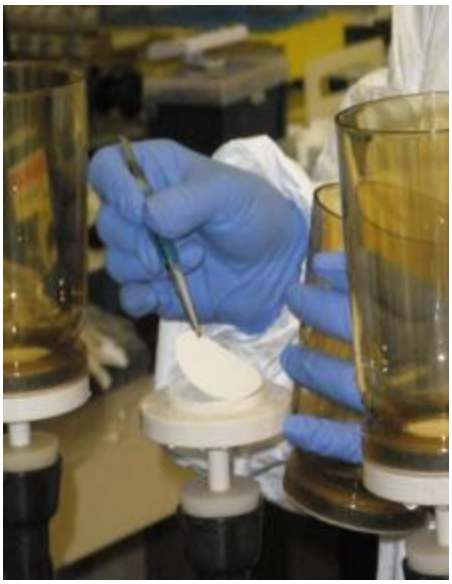

Figure 14. Filtration of water samples for bacterial water quality testing.

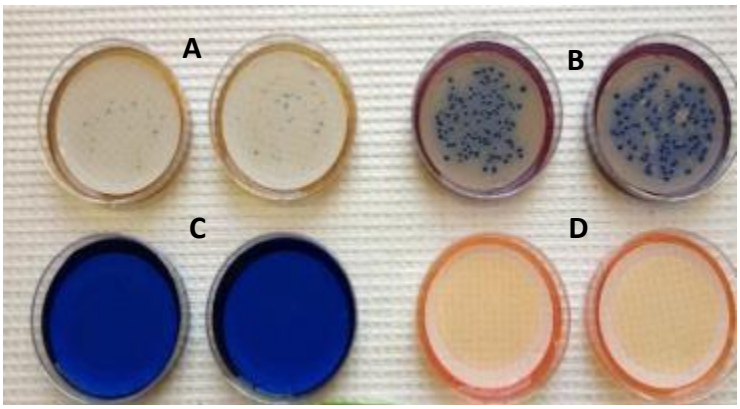

Figure 15. Use of selective media to enumerate bacteria of interest. Selective media and appropriate temperature are used to allow growth of the bacteria of interest. A=mEI for Enterococci; B=mFC for fecal coliforms; C=DTC for *Serratia marcescens*; D=MSA for *Staphylococcus* & *S. aureus*.

C. Sediment Porewater Toxicity Testing

1. Sediment Collection for Toxicity Testing

Collection of sediment samples for toxicity testing requires advanced preparation of sampling supplies and equipment. Two sampling methods were demonstrated during the training. The first was sediment collections using EPA pre-cleaned glass jars and the second was a coring technique that uses a modified syringe as a coring device and Teflon bags with closure clips to hold the sediment. Details of both techniques can be viewed in the CDHC training video (http://cdhc.noaa.gov/education/field_health.aspx).

The coring technique uses 30-60cc syringes without rubber or silicone tipped plungers (e.g., Fisher Scientific #03-377-24). They are first modified by removing their conical ends to create a cylinder to serve as a coring device. The modified syringes and Teflon bags are then cleaned according to EPA protocols (washed with Liquinox soap, rinsed 5-8 times with type I water and a final rinse with acetone) and packaged in acetone rinsed aluminum foil.

Figure 16. Cleaning divers and dive gear prior to sediment sampling for toxicity testing.

It is critical that sample collectors are free from any possible cross-contaminating substances (e.g., personal care products, sunscreens, etc.) which could confound the analyses. Therefore, immediately prior to sampling, sample collectors and their gear are cleaned with a standard laboratory detergent (e.g., Liquinox) prior to entering the water (Fig. 16). To further protect against cross-contamination, sample collectors and sample handlers must wear nitrile gloves during the collection and handling process, changing gloves between samples.

Figure 17. Sediment collection using a modified syringe technique and capping with nitrile gloves.

Surface sediment samples (containing porewater) are collected from target sites using EPA pre-cleaned glass jars or a modified syringe method (Downs *et al.* 2011; Woodley *et al*. 2013; see also training video for collection techniques: http://cdhc.noaa.gov/education/field_health.aspx). Modified syringe barrels are placed on the surface of undisturbed sediment with the plunger in. As the syringe is inserted into the sediment at an approximate depth of 3-5 cm, the plunger is slowly pulled, allowing sediment to fill the barrel. Nitrile gloves and a rubber band can be used to secure the sediment during transport to the surface (Fig. 17). Sediment is transferred from syringes to

PFA-Teflon bags (Welch Fluorocarbon, Dover NH), either at the surface or underwater (depending on the depth). Sufficient surface sediment should be collected to acquire 50 mL of porewater for the initial toxicity test or 500 mL for the TIE. Sediment can be stored for up to 48 hr at 4°C. Note that collection can only proceed if the surface sediment remains undisturbed in an area.

2. Extraction of Porewater from Sediment

Porewater can be extracted by centrifugation, gravity extraction, suctioned through an airstone attached to a syringe, or by glass pipet using a pipet-aid. Using a glass pipet and pipet-aid followed by centrifugation (1200 x g for 20 min) in glass or Teflon tubes was found to be an effective technique for extracting porewater from the sediment and removing sediment fines. Samples can be used immediately in a toxicity bioassay or placed in Teflon jars or bags, and frozen until analyzed (-20 °C to -80 °C).

C. Sea Urchin Development Toxicity Bioassay Training

Historically, porewater toxicity assays have been conducted with a number of test organisms that include polychaetes, benthic amphipods, fish embryos, copepods, microalgae and others (Carr and Nipper 2003). It is important to recognize that organisms vary in their sensitivity to different contaminants and confounding factors (e.g., ammonia, salinity) and should be taken into consideration when selecting test organisms. Using an appropriate bioassay test organism, to determine the quality of the sediments is a low-cost alternative to expensive analytical chemistry, with the added advantage of being able to detect biological effects of chemicals or mixtures at levels below detectability of standard analytical instruments.

Sea urchins have been recognized by EPA as useful indicators for environmental contamination because their sperm, embryos and larvae are very sensitive to toxicants. The sea urchin embryo development toxicity bioassay has become a generally accepted method (US EPA, Environment Canada, ASTM) for evaluating pollutant toxicity in marine waters. This bioassay is rapid, inexpensive, applicable to a variety of toxicants, and can be used to evaluate pollutant genotoxicity, embryo toxicity and teratogenicity. Sea urchins are part of tropical near-shore environments, thus are a relevant test organism for assessing the spatial distribution of water and benthic conditions toxic to reef organisms (including coral) in the coastal waters of Maui, Hawai‘i.

Prior to initiating the sea urchin development bioassay, porewater is tested for the following parameters: salinity, pH, unionized ammonia (NH_3), total ammonia nitrogen (TAN) and dissolved oxygen. These parameters should be adjusted to meet assay requirements based on known sensitivities of the species being used for the bioassay.

A detailed protocol for the sea urchin development bioassay can be found in Appendix III. Briefly, sea urchin gametes were obtained from *Tripneustes gratilla* for the class by shaking the urchins for up to

two minutes. Sperm were collected dry using a glass Pasteur pipet or micropipette and placed on ice until use. An aliquot of the dry sperm was diluted and checked microscopically for high motility and concentration. Eggs were collected over a beaker of seawater (Fig. 18) and inspected for proper shape and size. They were then washed and their concentration is adjusted and readied for fertilization. It is worth noting that other species (e.g., Arbacia, sand dollars, Lytechinus) have been used for this assay and have differing requirements relative to collecting gametes and assay requirements (e.g., salinity, temperature).

Eggs are fertilized with the addition of an appropriate amount of sperm (determined in a pre-test) and fertilization efficiency is determined by the percentage of eggs with fertilization membranes (see Fig. 19 development panel). Each porewater replicate receives 30-50 embryos/ml into the test vial to initiate the assay. The samples are incubated at 25°C (other urchin species may vary in incubation temperature requirements) and the test is terminated after controls reach pluteus stage (approx. 56 hours) with the addition of a formalin-based fixative. Embryos (100 count) are scored as normal or with degrees of abnormal development under 100X magnification. Percent normal development (normal plutei) in each treatment is compared to the reference treatment, artificial sea water control and ANOVAs are conducted to determine samples that are differ significantly ($p<0.05$) from controls, indicating positive toxicity of the sample.

Figure 18. Sea urchin egg collection. A female *Tripneustes gratilla* is inverted over a beaker filled with sea water. Eggs are negatively buoyant, thus sink to the bottom.

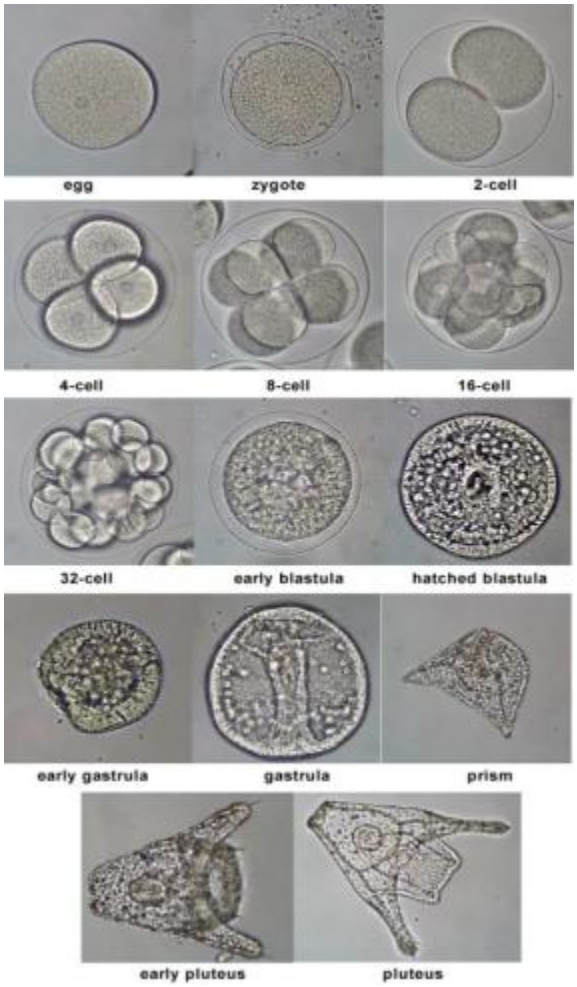

Figure 19. Sea urchin developmental staging series. Micrographs of *Lytechinus* sp. developmental stages from egg to pluteus larva. *Reproduced with permission from Dr. Cebra-Thomas. http://sites.millersville.edu/jcebrathomas/cebra_thomas/DB_lab/Urchin/urchin_stage.html*

Acknowledgments
We very much appreciate the funding support for this work (CRCP Project 502) provided by the Coral Reef Conservation Program to CMW.

We would like to thank all of the training workshop participants that took time to learn new skills as well as provide their experience and knowledge that helped define how the skills in bacterial water quality and using the sea urchin bioassay for toxicity testing were applicable to their management issues.

We especially thank those assisting with field collections. Assistance was provided in 2012 by Ka'au Abraham, Donna Brown, Meghan Dailer, Skippy Hau, Amy Hodges, Jonathan Martinez, Megan Ross, Hudson Slay and Robyn Watson. Assistance in 2013 was provided by Jonathan Martinez, Mark Miller and Wakea Po.

We wish to thank Athena Burnett for her contribution of artwork to this report.

We appreciate the work of Carl Miller for his aquaculture expertise and maintaining sea urchins at the Coral Culture Facility, NCCOS, Charleston, SC.

We give a special thanks to Dr. Paul Pennington (NCCOS, Charleston SC) for conducting statistical analyses for the toxicity testing data.

We appreciate the comments and corrections offered by the three internal reviewers, Jonathan Martinez, Laura Webster and Dave Whitall, which substantially improved the content of this report.

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Appendix I. Seawater Bacterial Water Quality Enumeration

Sampling plan: 8 L seawater from each site

- Kahekili, 2 m from seep
- Kahekili, 10 m from seep
- Kahekili, 20 m from seep
- Dead Zone 1
- Dead Zone 2
- Puamana
- Honolua Bay
- Kapalua
- Honokowai Beach Park
- La Perouse

Selection media

GASWA—100 ml sterile seawater in 1 L PP bottles (background control) (3)
MSA—2, 20 and 200 mL at each site in triplicate (9) *Staphylococcus* sp.
DTC—5, 50, and 500 mL at each site in triplicate (9) *Serratia marcescens*
mFC—5, 50 and 500 mL at each site in triplicate (9) Fecal coliforms
mEI—5, 50 and 500 mL at each site in triplicate (9) *Enterococcus* sp.

Equipment

1. Cooler for drinks, snacks (1)
2. Small cooler for fixed water samples (1)
3. Ice packs (for fixed water samples)
4. Marine cooler at ambient temperature for 6 L seawater samples (1)
5. 1L Nalgene bottles with lids (24)
6. Portable incubators (2)
7. Vacuum pumps and tubing (2)
8. 6-place vacuum manifolds (1)
9. 3-place vacuum manifold (1)
10. Traps for vacuum pump (2)
11. Extra filters for vacuum line (5)
12. Alcohol lamps (3)
13. Scripto lighters (3)
14. P1000 pipettor
15. P200 pipettor
16. P20 pipettor
17. P10 pipettor
18. Squirt bottle, 70% Isopropanol (2)
19. Squirt bottle, SASW (2)
20. Squirt bottle, 10% bleach (2)
21. Magnetic filter units (21)
22. 500 mL graduated cylinder (1)
23. Spray bottles (2)
24. Colony counter light box
25. Stir plate/magnetic stir bars
26. 2 L plastic beaker (2)
27. Mesh dive bags (4)

28. Digital pH meter
29. Thermometer
30. GPS
31. Waterproof camera with float (Cheryl's and the one in coral bldg)
32. Camera battery charger/extra batteries
33. Refractometer
34. First aid kit
35. Manual and automatic pipettors
36. Fine forceps (6-10)
37. Tool kit
38. Bucket
39. Rope (10 m)

Supplies

1. Gloves, small (1 box)
2. Gloves, medium (4 boxes)
3. Gloves, large (1 box)
4. Gloves, ex large (2 boxes)
5. Pipettes, 10 ml, for fixative (1 cs)
6. Pipettes, 50 ml, for SAWS (1 cs)
7. Pipette tips, 200 ul (4 boxes)
8. Pipette tips, 1000 ul (2 boxes)
9. Pipette tips, 20 ul (2 boxes)
10. Pipette tips, 10 ul (2 boxes)
11. 15 ml Falcon tubes – (2rack)
12. 50 ml Falcon tubes – (2 racks)
13. **mFC agar 60 mm plates
14. **Mannitol Salt agar 60 mm plates
15. **DTC agar 60 mm plates
16. GASW agar 60 mm plates
17. mEI agar 60 mm plates
18. Whatman GFF filters (800)
19. Nitrocellulose filters (800)—
20. 150 ml glass beakers for flaming (2)
21. Ethanol for alcohol lamps
22. Isopropanol
23. Bleach (will treat all biohazardous waste)
24. Sigma sea salts (2 kg)
25. MilliQ water source, or carboys of MilliQ – need to purchase distilled water on site
26. Refrigerator – use condo fridges
27. Electricity source – extension cords and power strips
28. 1 L Nalgene filter units (9)
29. Wheaton glass bottles, sterile 1L (12)
30. 500 ml bottle top filters (12)
31. Kimwipes
32. Paper towels
33. Parafilm
34. Versi-dry sheets

35. Garbage bags and ties
36. Lab markers, fine and broad tip
37. Lab tape
38. Packing tape
39. Scissors (2)
40. Aluminum foil
41. Field notebook /record sheets
42. 10 ml syringes
43. Clipboards (2-3)

Filtering amounts per day:

Seawater samples for direct plating onto selective media (filter using same three units beginning with smallest volume, in triplicate):

- mFC agar: 5, 50 and 500 mL (9) *Fecal coliforms*
- Mannitol Salts agar: 1, 5 and 10 mL (9) *Staphylococcus aureus*
- DTC agar: 5, 50 and 500 mL (9) *Serratia marcescens*
- mEI agar: 5, 50 and 500 mL (9) *Enterococcus*

Sterile seawater for "background" (3) on GASWA

Coral mucus samples for Staph: 10, 50 and 100 µL

Preparation Day 1:

1. Prepare 3 filter units with GFF and nitrocellulose filters (leave covered with aluminum foil). These will be used for filtering seawater samples immediately upon return.
2. Prepare enough sterile seawater (artificial) to do seawater filtering. Sterile seawater for filtering=~6 L/site + ~2 L sterile seawater for rinsing (8 L total).
3. Prepare and label selective media plates for filtering direct counts. Labels should read something like this: *Site 1*, *DTC, 200 ml*.
4. Set incubators at 41 ^{o}C and 35 ^{o}C (check with thermometer).
5. Rinse 8 1L Nalgene bottles three times with ~50 ml 70% isopropanol, leaving last rinse in bottle.
6. Pack field gear into waterproof containers for transport to sampling site.

Pack Field Gear for site sampling:

7. Cooler for ambient temp seawater (1)
8. Cooler for snacks and drinks (1)
9. GPS
10. Refractometer
11. Thermometer
12. pH meter
13. Field notebook
14. 70% Isopropanol spray bottle (1)
15. Gloves, one box each of small, medium and large , extra large (4)
16. Snorkel gear
17. Cameras (coral building and Cheryl's)
18. Towels
19. Sterile 1L Nalgene bottles (8)

Field Methods:

1. One person takes field measurements and records: time, tide, air/water temp, weather, salinity, lat/long.
2. One to two people set up processing station on boat/shore: coolers, notebook, etc.
3. Partially fill small marine cooler with ambient temperature seawater.
4. Take 8 1L samples of seawater from appropriate depth/site, making sure to completely rinse out any residual 70% isopropanol.
5. Store 1L seawater samples in marine cooler (shaded) containing ambient seawater.
6. Return to laboratory.

Laboratory Methods:

1. Set up 3 sterile filter units on 3-place vacuum manifold attached to trap/pump.
2. Sterilely place GFF and nitrocellulose filters on units and keep covered until ready to filter.
3. We will be filtering seawater samples from lowest volume to highest volume required for each type of media, and in triplicate, as indicated in the table below. For volumes less than 100 ml, add enough SASW for a total filtering volume of ~50 ml, prior to adding the natural seawater and vacuum filtering. Measure all sample volumes accurately with a pipette.
4. Prior to filtering, check to make sure all vacuum lines are closed.
5. For the first sample, add ~100 ml sterile artificial seawater (SASW) to each filter unit.
6. Swirl 1L sample bottle. Remove 0.2 ml aliquots of seawater and place each into separate filter units (as indicated in row 1 in the table below).
7. Once the natural seawater sample has been added to the filter unit, swirl gently with the pipette tip to mix.
8. Turn on the vacuum pump and open the valves for each filter unit.
9. When the liquid has been completely filtered, close the valves, rinse the sides of each filter unit with SASW well, and open valves to finish the filtering process.
10. Turn off pump.
11. Remove nitrocellulose filter from the filter unit (sterile technique!) and place on plate of selective agar (60 mm). Ensure that no air bubbles are trapped between filter and agar.
12. Rinse units with SASW and replace with new sterile nitrocellulose filter. GF filters can be reused if filtering samples from the same site. However, if seawater filtering rate slows, change the GF filter.
13. Repeat the process for the remaining seawater samples as detailed below, making sure to follow the order of media volumes exactly (filtering from lower to higher volumes).
14. **NOTE: for filtering 200-500 ml volumes, if water is very turbid, it may not be possible to filter the entire amount. Filter as much as possible using aliquots from a 50 ml pipette. An accurate account of how much seawater is filtered must be recorded.**
15. Place DTC, mEI, and mFC plates in 41 ^{o}C incubator and other plates at ambient temperature or 35^{o}C incubator.
16. Record CFU/plate after 24 h incubation based on appropriate color of colonies for each medium; photograph plates.

	Selective media	SASW to be added to each unit	Filter unit A	Filter unit B	Filter unit C	Total water (mL)
1	**GASWA**	**100 mL from sterile 1L bottle**	**------**	**------**	**------**	
2	MSA	~100 ml	2 mL	2 mL	2 mL	6
3	DTC	95 ml	5 ml	5 ml	5 ml	15
4	mFC	95 ml	5 ml	5 ml	5 ml	15
5	mEI	95 ml	5 ml	5 ml	5 ml	15
6	MSA	80 ml	20 ml	20 ml	20 ml	60
7	DTC	50 ml	50 ml	50 ml	50 ml	150
8	mFC	50 ml	50 ml	50 ml	50 ml	150
9	mEI	50 ml	50 ml	50 ml	50 ml	150
10	MSA	-------	200 ml	200 ml	200 ml	600
11	DTC	-------	500 ml	500 ml	500 ml	1500
12	mFC	-------	500 ml	500 ml	500 ml	1500
13	mEI	-------	500 ml	500 ml	500 ml	1500

NOTE: Once target range for bacterial selection has been determined, it will be possible to reduce the number of seawater dilutions performed at each site.

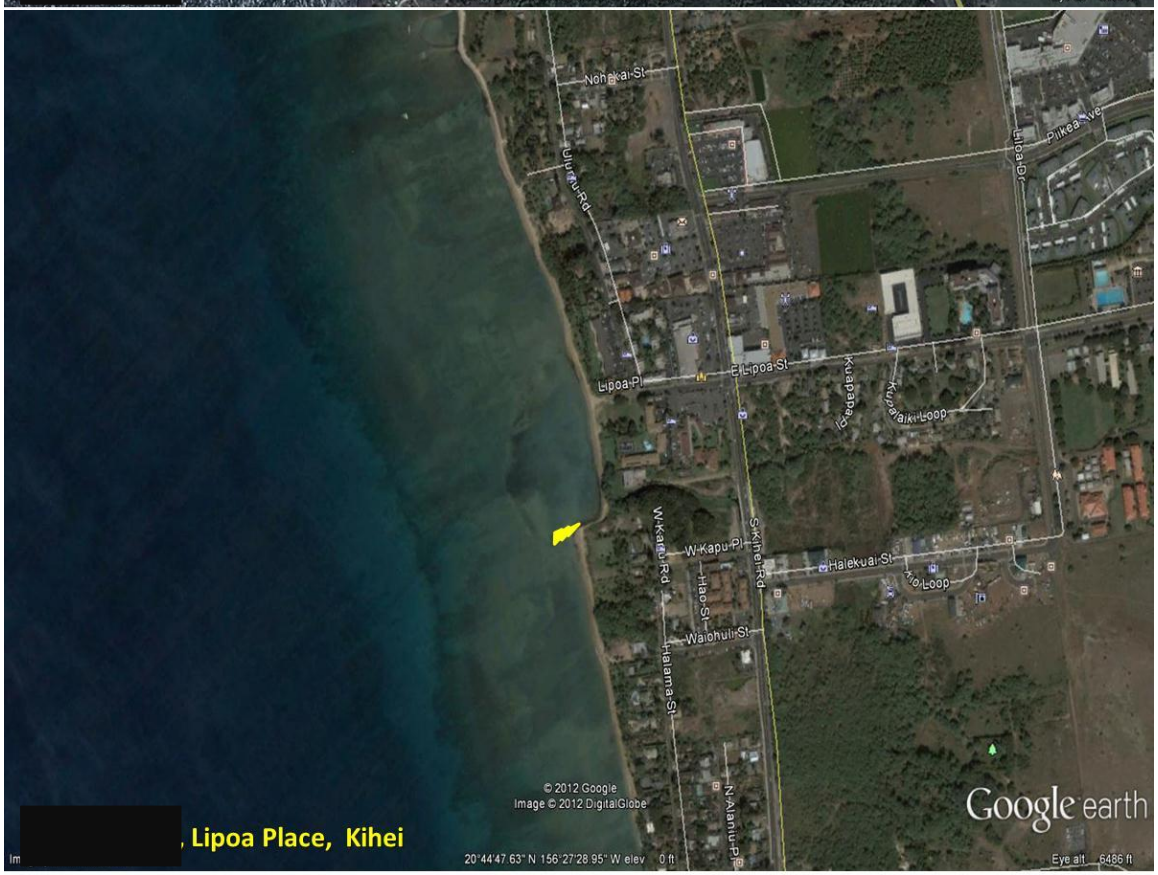
Appendix II. September 2012 Sediment Sampling Points along the Maui Coast

The following Google Earth 16 figures provide aerial images of each sampling site and indicates the specific location within the site that samples were taken.

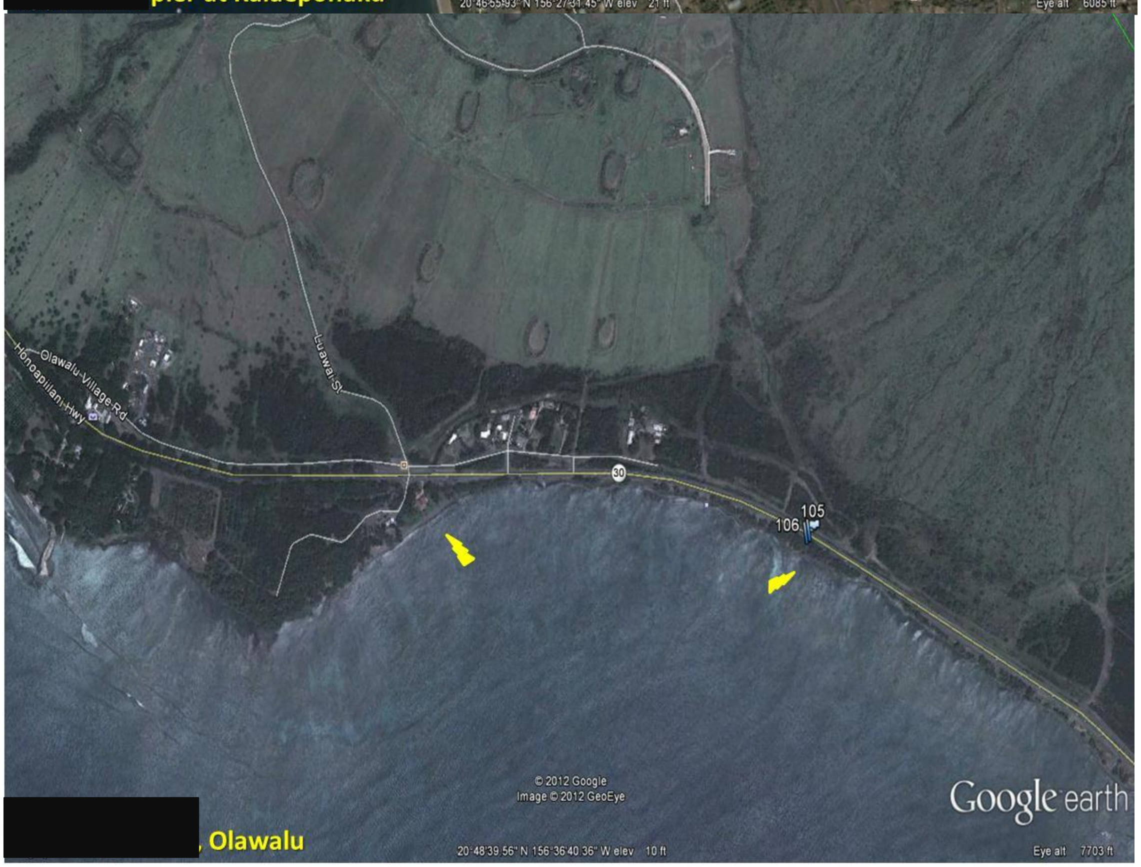

Makena Park
Makena Rd
Image © 2012 DigitalGlobe
© 2012 Google
20°37'59.94" N 156°26'52.14" W elev 0 ft
Eye alt 7503 ft
Google earth


Polo Beach
Polo Beach Club
A Plus Resorts
Fairmont Kea Lani
Wailea Alanui Dr
Kaukahi St
Manalo St
Wailana Pl
Waiola Loop
Image © 2012 DigitalGlobe
© 2012 Google
20°40'34.48" N 156°26'29.85" W elev 71 ft
Eye alt 4310 ft
Google earth


Ulua Beach Park
Ulua Beach Rd
Hale Alii Pl
Hale Hookipa Way
Wailea Alanui Dr
Wailea Ike Pl
Image © 2012 GeoEye
© 2012 Google
20°41'27.57" N 156°26'33.10" W elev 26 ft
Eye alt 2486 ft
Google earth


Lipoa Place, Kihei
Nohokai St
Uluniu Rd
Lipoa Dr
Pilkea Ave
Lipoa Pl
E Lipoa St
Kuapapa Pl
Kuapaki Loop
W Kapu Pl
Halekuai St
Waiohuli St
Halama St
N Alaniu Pl
© 2012 Google
Image © 2012 DigitalGlobe
20°44'47.63" N 156°27'28.95" W elev 0 ft
Eye alt 6486 ft
Google earth

[redacted] pier at Kalaepohaku



[redacted], Olawalu


[redacted], Lahaina
Kapunakea St
Keawe St
Ulupono St
Kupuohi St
Ala Moana St
Front St
Keaoi St
Nahale St
Hiki Pl
30
© 2012 Google
Image © 2012 DigitalGlobe
© 2012 Europa Technologies
Google earth


[redacted] Kahana Bay
Kili Nahe St
Hanawai St
Alaalo Pl
Napilihau St
Hui Rd B
Hui Rd C
Hui Rd E
Lower Honoapiilani Rd
30
© 2012 Google
Image © 2012 DigitalGlobe
© 2012 Europa Technologies
Google earth
Imagery Date: 1/25/2007


Honokeana Bay
Lower Honoapiilani Rd
Papaua Pl
Hui Rd F
© 2012 Google
Image © 2012 GeoEye
© 2012 Europa Technologies
Google earth
[redacted]
Honokeana Bay


Napili Bay
Lower Honoapiilani Rd
Hui Rd F
© 2012 Google
Image © 2012 GeoEye
© 2012 Europa Technologies
Google earth
[redacted]
Napili Bay


Namalu Bay
Napili Bay
Bay Dr
Ironwood Ln
Ridge Rd
Lower Honoapiilani Rd
Coconut Grove Ln
Kapalua Dr
Pineapple Rd
Cook Pine Rd
Hui Rd H
Image © 2012 DigitalGlobe
© 2012 Google
Google earth
[redacted], Kapalua Bay
20°59'57.14" N 156°39'49.29" W elev 91 ft
Eye alt 4867 ft


30
Kai Ala Dr
Honoapiilani Hwy
Image © 2012 GeoEye
© 2012 Google
Google earth
[redacted], Near North Seep
20°56'19.32" N 156°41'27.19" W elev 9 ft
Eye alt 2478 ft

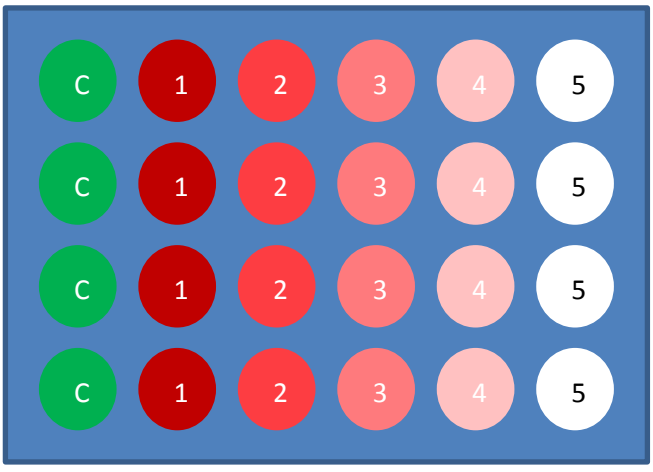
Appendix III. Sea Urchin Embryo Toxicity Test Protocol

Materials:

- Sea urchin embryos (single-cell zygote)
- Sedgewick-Rafter counter
- Teflon 24-well plate or appropriate number of Teflon vials for dosing (minimum of 4 replicates per dose, including control)
- Micropipettor, 1000 µL
- Micropipettor, 200 µL
- Teflon micropipette tips, 1000 µL
- Teflon micropipette tips, 200 µL
- Glass pipets, 10 mL
- Glass Pasteur pipets
- Transfer pipets
- Teflon jars 60ml for TIE collection
- Sediment porewater samples, or chemical compound of interest diluted in sterile seawater
- Solvent for compound dilution in seawater, if required
- Incubator (set at temperature appropriate for embryo growth)
- Microscope (10x-40x objectives) with imaging capability
- Filtered seawater
- Formalin, 10% solution in seawater
- Adjust pH if necessary
- Acid & base solutions and what are they?
- Concave slides

1. Prepare the test solutions, and then fill the appropriately labeled testing chambers with the test solution using micropipettors with Teflon tips (see below). Use a single Teflon tip per test solution. There should be at 4 replicates per test treatment, including for the sea water control and a carrier solvent control if a carrier solvent was used. When calculating the test solution concentration, you should factor into your calculation that you will be adding 100 µL of zygote solution to the final test volume.

Treatment C: control, seawater (Green)
Treatment 1: Porewater 1
Treatment 2: Porewater 2
Treatment 3: Porewater 3
Treatment 4: Porewater 4
Treatment 5: Porewater 5



2. Dilute or concentrate the zygotes to approximately 30 zygotes per 100 µL.

3. The final test volume in each test chamber should not exceed 15 zygotes per milliliter. It is recommended that you use a 2 mL test chamber, so that each chamber should hold about 30 zygotes.
4. It is acceptable to have more or less than 30 zygotes per chamber; provided the minimum number of zygotes is 20 and the maximum is 40.
5. Add zygotes to the test chambers.
6. Examine each test chamber for the number of fertilized zygotes per total number of sea urchin egg/zygotes of each chamber. This is a quality control step to ensure a baseline for actual zygotes in each test chamber.
7. The exposure period can last up to 48 hours. Embryos should be examined with a microscope every 12 hours, tabulating the number of healthy looking developing embryos versus the number of those that are deformed. If possible, photo-document each replicate at each time point. A wide-field (low magnification; 4x or 10x) image should be taken, and a higher-magnified (40x or 100x) image should also be taken.
8. At the end of the exposure, development can be stopped by adding 400 µL of 10% formalin/seawater to the dosing vessel. This will preserve the embryos for at least 2-4 days to allow for more detailed observations of individual embryos or larval forms in each test chamber.
9. Using a microscope, count the number of deformed embryos and describe and photo-document the dominant deformation morphology.

APPENDIX IV. 2012 KAHEKILI SAMPLES: CLASSES OF SEA URCHIN (*ARBACIA PUNCTULATA*) DEVELOPMENTAL ABNORMALITIES FROM POREWATER TOXICITY TESTING

SAMPLE	% NORMAL	SE	% UNDER DEVELOPED	SE	% MALFORMED	SE
CN	99.5	± 0.3	0.3	± 0.3	0.3	± 0.3
SOS	51.9	± 6.4	45.0	± 6.4	3.0	± 0.4
WDA	84.8	± 3.0	9.0	± 2.0	6.3	± 1.4
WDB	53.0	± 5.6	31.7	± 3.9	15.3	± 7.6
WLA	13.1	± 3.4	56.9	± 3.5	30.0	± 6.4
WLB	92.8	± 0.9	6.0	± 0.7	1.3	± 0.5
NEG CONTROL	90.5	± 1.3	5.5	± 1.3	4.0	± 0.7
POS CONTROL	67.8	± 3.2	29.8	± 2.1	2.5	± 2.2
	SE=STANDARD ERROR OF THE MEAN					

APPENDIX V. 2013 KAHEKILI SAMPLES: CLASSES OF SEA URCHIN (*ARBACIA PUNCTULATA*) DEVELOPMENTAL ABNORMALITIES FROM POREWATER TOXICITY TESTING

SAMPLE	% NORMAL	SE	% UNDER DEVELOPED	SE	% ARRESTED DEVELOPMENT	SE	% MALFORMED	SE
RUNWAY 6/25/13	0.0	± 0.0	7.5	± 2.5	0.0	± 0.0	92.5	± 2.5
BONE YARD 6/25/13	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0	100.0	± 0.0
SOUTH SEEP 6/25/13	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0	100.0	± 0.0
BLACK ROCK 6/25/13	67.0	± 1.5	2.3	± 0.9	5.5	± 1.0	25.3	± 2.6
RUNWAY 6/30/13	47.5	± 4.1	16.2	± 3.1	4.0	± 0.7	32.3	± 4.1
BONE YARD 6/30/13	59.2	± 4.1	4.7	± 0.8	9.0	± 2.0	27.2	± 3.3
SAND CHANNEL 6/30/13	67.8	± 3.0	3.8	± 1.5	3.5	± 1.0	25.0	± 1.4
NEG CONTROL	64.8	± 2.1	2.0	± 0.7	6.5	± 2.0	26.8	± 2.3
POS CONTROL	15.5	± 2.4	28.6	± 2.9	3.9	± 2.3	51.9	± 5.4
	SE=STANDARD ERROR OF THE MEAN							

APPENDIX VI. 2012 OTHER MAUI SITES: CLASSES OF SEA URCHIN (*LYTECHINUS VARIEGATUS*)

Developmental Abnormalities from Porewater Toxicity Testing

Sample	% normal	SE	% arrested Development	SE	% deformed	SE	% dead	SE
Kapalua Bay North	2.0	2.0	14.9	4.3	68.0	4.4	15.1	2.6
Kapalua Bay South	0.0	0.0	46.1	5.3	35.3	4.7	18.6	2.6
Napili Bay	51.1	10.1	28.4	6.5	16.3	3.1	4.2	1.6
Honokeana Bay	0.0	0.0	0.0	0.0	50.4	4.6	49.6	4.6
Kahana Bay	50.1	7.1	22.5	3.2	27.4	5.8	0.0	0.0
Lahaina	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0
Olowalu North	73.4	4.6	0.0	0.0	18.9	3.7	7.6	1.6
Olowalu South	80.9	2.3	0.0	0.0	17.6	1.7	1.5	1.5
Pier at Kalaepohaku	0.0	0.0	73.4	7.5	5.1	3.2	21.5	5.3
Lipoa Place	39.0	10.1	18.3	2.8	23.9	3.8	18.7	4.0
Ulua Beach Park	92.0	3.6	0.0	0.0	7.0	2.9	1.0	1.0
Wailea, Polo Beach	93.9	2.8	0.0	0.0	5.2	2.2	0.8	0.8
Makena Park, Little Beach	91.7	2.8	2.8	0.9	3.8	1.6	1.8	1.0
Makena Road	93.4	0.9	0.0	0.0	5.6	0.9	1.0	1.0
La Perouse	99.2	0.8	0.0	0.0	0.0	0.0	0.8	0.8
<u>Kahekili Samples</u>								
WNA	96.3	1.4	0.0	0.0	3.7	1.4	0.0	0.0
South of South Seep	93.8	2.5	0.0	0.0	5.6	2.2	0.6	0.6
North Seep	81.3	3.5	0.0	0.0	10.2	3.5	8.5	1.9

SE=standard error of the mean

Appendix VII. 2013 Kahekili Samples: Classes of Sea Urchin (*Arbacia punctulata*) Developmental Abnormalities from Porewater Toxicity Testing

Sample	% Normal	SE	% Retarded development	SE	% Arrested Development	SE	% Malformed	SE
Honolua Bay	58.6	± 1.8	3.8	± 1.4	4.1	± 0.5	33.5	± 2.4
Kapalua North	51.4	± 2.7	21.5	± 1.5	3.7	± 0.9	23.4	± 3.4
Kapalua South	34.8	± 6.9	35.8	± 7.7	2.5	± 0.6	27.0	± 3.5
Napili	67.3	± 4.3	1.3	± 0.5	3.5	± 1.4	28.0	± 4.7
Honokeana Cove	61.7	± 4.1	4.6	± 1.4	4.8	± 1.0	28.9	± 3.8
La Perouse	70.4	± 5.8	0.3	± 0.3	13.7	± 2.6	15.6	± 5.7
Neg Control	64.8	± 2.1	2.0	± 0.7	6.5	± 2.0	26.8	± 2.3
Pos Control	15.5	± 2.4	28.6	± 2.9	3.9	± 2.3	51.9	± 5.4

SE=standard error of the mean

APPENDIX VIII. 2012 MAUI SAMPLES: PHASE I TOXICITY IDENTIFICATION EVALUATION (TIE) ANALYSIS EVALUATING TOXICITY REDUCTION USING SEA URCHIN (*LYTECHINUS VARIEGATUS*) DEVELOPMENTAL BIOASSAYS

	Treatment	% Normal ±SE	% Arrested ±SE	% Deformed ±SE	% Dead ±SE
KAPALUA BAY NORTH					
	Porewater Toxicity	2.0 ±2.0	14.9 ±4.3	68.0 ±4.4	15.1 ±2.6
	C-18	**83.2 ±2.3**	**0.0 ±0.0**	**32.2 ±14.6**	**0.8 ±0.8**
	Verify AX	**48.5 ±11.3**	**0.0 ±0.0**	**51.5 ±11.9**	**0.0 ±0.0**
NORTH SEEP KAHEKILI					
	Porewater Toxicity	81.3 ±3.5	0.0 ±0.0	10.2 ±3.5	8.5 ±1.9
	C-18	97.0 ±3.0	0.0 ±0.0	0.0 ±0.0	3.0 ±3.0
	Verify AX	**100.0 ±0.0**	**0.0 ±0.0**	**0.0 ±0.0**	**0.0 ±0.0**
OLOWALU NORTH					
	Porewater Toxicity	80.9 ±2.3	0.0 ±0.0	17.6 ±1.7	1.5 ±1.5
	C-18 **	80.0	0.0	20.0	0.0
	Verify AX	**…**	**…**	**…**	**…**
OLOWALU SOUTH					
	Porewater Toxicity	73.4 ±4.6	0.0 ±0.0	18.9 ±3.7	7.6 ±1.6
	C-18 **	**100.0**	0.0	**0.0**	**0.0**
	Verify AX	**…**	**…**	**…**	**…**
PIER AT KALAEPOHAKU					
	Porewater Toxicity	0.0 ±0.0	73.4 ±7.5	5.1 ±3.2	21.5 ±5.3
	C-18	**90.5 ±2.1**	**9.5 ±2.1**	**0.0 ±0.0**	**0.0 ±0.0**
	Verify AX	**96.1 ±2.0**	**1.7 ±1.7**	**0.0 ±0.0**	**2.2 ±2.2**
HONOKEANA BAY					
	Porewater Toxicity	0.0 ±0.0	0.0 ±0.0	50.4 ±4.6	49.6 ±4.6
	C-18	**77.1 ±2.9**	0.0 ±0.0	**21.0 ±1.0**	**1.9 ±1.9**
	Verify AX	**…**	**…**	**…**	**…**
LIPOA PLACE					
	Porewater Toxicity	39.0 ± 10.1	18.3 ±2.8	23.9 ±3.8	18.7 ±4.0
	C-18	48.2 ±1.9	19.1 ±2.4	32.7 ±4.4	0.0 ±0.0
	Verify AX	**100.0 ±0.0**	**0.0 ±0.0**	**0.0 ±0.0**	**0.0 ± 0.0**

SE= standard error of the mean
**denotes only one replicate for the bioassay because of sample limitation
…=treatment not conducted
Red text indicates notable toxicity reduction in various developmental abnormalities

APPENDIX IX. PRELIMINARY TOXICITY IDENTIFICATION EVALUATION PHASE II

SPE columns for three samples, SS, South of the South Seep, and Kapalua North, that reduced toxicity (i.e., binding toxicant) were analyzed by Jupiter Labs for contaminant chemistry screening. The samples arrived frozen and in good condition and were stored below zero until extraction. All samples we run using the AB SCIEX 5500 Q-Trap with blanks and controls to insure the quality of the data.

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Analyte	ssb3 strata	ssb3 c18	ssb3 ax	Kapalua AX	Kapalua C18	scep c18	scep ax
Acetaminophen 1							
Caffeine 1	x						
carbamazepine 1	x	x	x			x	x
ciprofloxacin hcl 1							
diclofenac sodium salt 1							
erythromycin 1							
fluoxetine 1							
primidone 1	x	x	x				
progestrone 1							
sulfamethoxazole 1	x	x	x				
testosterone 1							
Trimethoprim 1							
2,4,5T 1							
2,4,5TP 1							
2.4-D 1							
2.4-DB 1							
3,5-dichlorobenzoic acid 1							
4 nitrophenol 1							
Acifluorfen 1							
Bentazon 1							
dalapon 1							
Dicamba 1							
dichloroprop 1							
Dinoseb 1							
MCPA 1							
Mecoprop-P 1							
PCF 1					x		
picloram 1							
p tert amyl phenol 1							
4 tert butylphenol 1							
bis a 1							
ESTRONE 1							
Nonylphenol 1							
4 tert octylphenol 1							
17 a ethynylestradiol 1							
17 b Estradiol 1							
1 bromo 2 nitro benzene 1			x				
aspon 1							
Atrazine 1		x			x	x	
azinophos methyl Guthion 1							
azinophos-ethyl 1							
carbophenothion 1							
chlorfenvinphos 1							
chlorpyriphos 1							
chlorpyriphos- methyl 1							
crotoxyphos 1							
cumaphos 1							
diazinon 1							
dichlorofenthion 1							
dichlorvos 1							
dichrotophos 1							
dimethoate 1							
dioxathion 1							
disulfoton 1							
epn 1							
ethion 1							
ethoprop 1							
ethyl parathion 1							
famphur 1							
fenchlorphos ronnel 1							
fenitrothion 1							
fensulfothion 1							
fenthion 1							
fonophos 1							
leptophos 1							
malathion 1					x		
merphos 1							
m parathion 1							
mevinphos 1							
monocrotophos 1							
naled 1							
phorate 1							
phosmadion 1							
phosmet 1							
Simazine 1						x	
sulfotepp 1							
sulprofos Bolstar 1							
Teep 1							
terbufos 1							
tetrachlorvinphos 1							
thionazin 1							
tokuthion 1							
trichlorfon 1							
trichloronate 1							

"X" indicates presence of contaminant