EXHIBIT CC

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
REGION IX
75 Hawthorne Street
San Francisco, CA 94105-3901

March 24, 2008

Lorilee Crisostomo
Administrator
Guam Environmental Protection Agency
P.O. Box 22439
Barrigada, Guam 96921

Dear Ms. Crisostomo:

Our agencies recently had a conference call to discuss mixing zones related to discharges from the Guam Water Authority's (GWA's) Northern District and Agana wastewater treatment facilities. During the conference call, your staff indicated that they have concerns with the ability of the discharges to meet water quality standards and that additional treatment may be necessary at these facilities. We then discussed the requirements for state certifications related to 301(h) applications. I agreed to provide additional explanation of the certification procedures and to share EPA Region 9's current understanding on whether the proposed discharges from these facilities are likely to meet Guam's water quality standards for enterococcus bacteria. This letter contains the information we agreed to provide and requests your decisions on certification.

The Clean Water Act generally requires that publicly owned treatment works provide secondary treatment to wastewater that is discharged to waters of the United States. Section 301(h) of the Clean Water Act provides for an exception to this general requirement, if the discharger demonstrates to the satisfaction of EPA, with the concurrence of the state, that certain requirements are met. EPA Region 9 has received applications for variances in accordance with Section 301(h) from GWA for the Northern District and Agana facilities.

EPA has promulgated regulations that govern the review of Section 301(h) applications, several of which pertain to the requirement for state concurrence:

- 40 CFR 124.54(a) provides that when an application for a permit incorporating a Section 301(h) variance is submitted to a state, the appropriate state official shall either deny the request for the 301(h) variance (and so notify the applicant and EPA) or forward a certification in accordance with 40 CFR 124.53 and section 401 of the Clean Water Act and 40 CFR 124.53. For the proposed Guam discharges, this certification pertains to whether the proposed discharge will meet requirements related to total suspended solids and biochemical oxygen demand.

- 40 CFR 125.59(b)(3) provides that no Section 301(h) variance can be issued where issuance would conflict with applicable provisions of state, local or other Federal laws.

- 40 CFR 125.61(b)(2) provides that the Section 301(h) applicant must provide a determination signed by the state that the proposed discharge will comply with applicable provisions of state law, including water quality standards (this determination shall include a discussion of the basis for the conclusion reached).

- 40 CFR 125.64(b) provides that the applicant must obtain a determination from the state indicating whether the applicant's discharge will result in an additional treatment pollution control, or other requirement on any other point or nonpoint sources (the state determination shall include a discussion of the basis for its conclusions).

Thus, there are multiple opportunities for the Guam Environmental Protection Agency to provide certification or approval associated with the Section 301(h) application process. As your staff expressed concern about the likelihood that the proposed discharges would meet water quality standards, our discussion focused on the certification required by 40 CFR 125.61(b)(2). This certification is separate from state approval of a request for a mixing zone under state water quality standards, in this case 22 Guam Administrative Rules (GAR) Section 5104.

EPA Region 9 is reviewing the two GWA applications. Both applications are for facilities with primary treatment and extended ocean outfalls, without disinfection. Although we are in the initial phases of our review, our assessment at this time is that it is unlikely either of the proposed discharges would be able to meet Guam's water quality standards for enterococcus bacteria. This assessment is based on studies conducted and/or data compiled by GWA, as described in their application, and as described in the engineering and technical references on the performance of primary treatment plants.

The proposed discharges are into waters designated as M-2 in Guam's water quality standards. GAR Section 5102 identifies propagation and survival of marine animals and primary recreation as designated uses for these waters. GAR Section 5103(C)(1) specifies that the following criteria apply:

> "Concentrations of enterococci bacteria shall not exceed 35 enterococci /100ml based upon the geometric mean of five (5) sequential samples taken over a period of thirty (30) days. No instantaneous reading shall exceed 104 enterococci /100ml."

GWA states, in the basis of design reports for both ocean outfall extensions, a dilution of up to 8000 would be required to meet the enterococcus criteria. However, the outfall for the Northern District facility is only designed to attain an initial dilution of 200 and the outfall for the Agana facility is only designed to attain a dilution of 100. Thus, the anticipated dilution for both outfalls is not sufficient to meet the water quality standard.

This analysis is also supported by technical literature on wastewater treatment. Miescier and Cabelli found that primary treatment decreased enterococci densities by about 25% (Miescier, J.J. and Cabelli, V.J. 1982. Enterococci and other microbial indicators in municipal wastewater effluents. Journal of Water Pollution Control Federation, 54:1599-1606). Primary treatment alone, therefore, does not reduce bacteria levels to the extent required by Guam's water quality standards. GWA's basis of design reports for the two outfalls project an effluent value of 830,000 enterococci/100ml after primary treatment, which is significantly higher than the 104 enterococci/100 ml standard, even when accounting for initial dilution.

Given the requirements for state concurrence and our initial analysis of the 301(h) applications, we would like to request your determination under 40 CFR 125.61(b)(2) for the Northern District and Agana facilities. Your determination should be submitted to EPA via letter to Ms. Alexis Strauss, Director of the Water Division. The letter must include the specific grounds for granting or denying the certification, including the specific statutory or regulatory provisions at issue (e.g., GAR Section 5104(A)(9)). If you grant certification, we will continue with our analysis of the application and prepare a Tentative Decision Document. If you deny certification, we will request that GWA submit applications for a permit for secondary treatment. We would appreciate receiving your determinations as soon as possible, so we know which path to pursue, as we are committed to completing the tentative decisions on the 301(h) applications this summer. We understand that this is a complicated process and would gladly discuss it with your staff as needed.

If you have any questions regarding the requirements of Section 301(h) or our preliminary assessment, please contact me at 415-972-3420 or Michael Lee, Pacific Islands Office, at 415-972-3769.

Sincerely,

Douglas E. Eberhardt

Douglas E. Eberhardt
Chief, NPDES Permits Office

cc: M. Gawel, GEPA
M. Minas, GEPA
B. Cruz, GEPA
J. Benavente, GWA
D. Antrobus, GWA

EXHIBIT DD

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
REGION IX
75 Hawthorne Street
San Francisco, CA 94105

November 27, 2007

Paul J. Kemp
Assistance General Manager for Compliance and Safety
Guam Waterworks Authority
P.O. Box 3010
Hagatna, Guam 96921

Re: Agana and Northern District STPs Operational Performance Evaluations
Stipulated Order Paragraphs 39 and 42

Dear Mr. Kemp:

This is a response to your letter dated November 16, 2007, requesting EPA to advise GWA on the type of information needed to satisfy the requirements of paragraphs 39 and 42 of the Stipulated Order (SO).

The operational performance evaluation (OPE) should be of sufficient nature to determine whether the sewage treatment plants can comply with current NPDES permit effluent limitations and whether there is a need for advance primary treatment. The OPE shall include, but not limited to, the following:

1. Sampling of the influent and effluent for flow, BOD, TSS, and settleable solids. Influent and effluent BOD, TSS and settleable solids for each of the primary clarifiers should be performed to determine the performance of each unit to ensure units are performing as expected based on their design characteristics and loadings. Flow measurement should correlate with BOD and TSS monitoring.

2. Sampling frequencies should be sufficient to determine the performance of each unit process and the quality of combined effluent. It is recommended that the sampling frequency be performed anywhere from three times per week to daily. Sampling may need to be adjusted based on evaluation of results during the performance evaluation period.

3. As per the SO, the OPE would be performed over a two month period.

4. Sampling should be 24 hour composites and located to ensure that representative samples are collected. All sampling locations should be identified and noted on a facility diagram.

5. All sampling results should be presented in a table that clearly shows the performance characteristics of the unit processes, treatment plant overall and comparison to NDPES permit effluent limitations. For the final effluent BOD and TSS, both concentrations and mass loadings

should be determined and compared to NPDES permit effluent limitations. Percent removal BOD and TSS should also be determined and included with the final results. Data from Discharge Monitoring Reports (DMR) should also be used as part of the OPE and to support the determination.

6. If the treatment plant is having difficulty meeting NPDES permit effluent limitations and the primary clarifiers are performing satisfactorily, then GWA may need to evaluate other treatment process as they may be contributing to the facility's inability to comply with permit effluent limitations.

Based on the OPE results, GWA shall submit a determination, in writing, supported with the OPE results/data, that the treatment plant is either able or not able to meet current NPDES permit effluent limitations (primary treatment) requirements.

If, based on the OPE results, it is determined that the treatment plant can meet current NPDES permit effluent limitations and no additional treatment is needed, then GWA has satisfied paragraphs 39 and 42. If it is determined that advanced primary treatment is needed, GWA shall include a schedule for any necessary design work and installation of a advance primary treatment system.

If you have any questions, please contact me at 415-972-3769 or at lee.michael@epa.gov.

Sincerely,

Michael J. Lee
Pacific Islands Office

cc: J. Benavente, GWA
D. Antrobus, GWA
L. Crisostomo, GEPA
M. Minas, GEPA

EXHIBIT EE

A. COVER PAGE

(1) Project Title: Testing for Links Between Nutrient Pollution and Coral Health and Disease

(2) Applicant Organizations: University of Guam and American University

(3) Principal Investigator: Dr. Laurie Raymundo
Associate Professor

(4) Contact Info: University of Guam Marine Laboratory
UOG Station
Mangilao, GU 96923
T (671) 735-2184, F (671) 734-6767 **ljraymundo@gmail.com**

Co-PI: Dr. Kiho Kim
Associate Professor
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(5) Program Priorities: Habitat Conservation

(6) Local Action Strategies: Coral Disease and Land-Based Pollution

(6) Geographic Location: Guam

(7) Funds Requested: $50,000

(8) Amount of Matching: Under 48 U.S.C., 1469 a(d), Guam is an Insular Area and University of Guam is not, therefore, not subject to the requirement to provide matching funds. However, $34,900 in-kind contributions from the Raymundo lab are provided toward this project.

(9) Start and End date: Jan 01, 2009 – June 30, 2010

(10) Project Summary: Guam currently has two primary treatment plants that release nutrient-rich waste water into coastal waters. Plans to upgrade these plants to secondary treatment have not been acted upon, though the human population is expected to increase greatly over the next five years. Effects of nutrient inputs on coral communities have not been addressed. The proposed project will document the impacts of sewage nutrient pollution to coral health and disease. We will address this problem by quantifying spatial and temporal changes in: 1) coral species diversity and cover at increasing distances from outfalls; 2) seasonal shifts in stable N isotopes and water quality at increasing distances from outfalls; and 3) coral disease prevalence and incidence, and progression and transmission rates, via field surveys and laboratory experiments, respectively. N stable isotope analyses of benthic fauna and flora will examine sources and spatial and temporal patterns of nutrient pollution, correlated with shifts in coral community composition and water quality. A nutrient dosing experiment will be carried out on diseased and healthy

corals, to examine the effect of elevated nutrients on the rate at which disease progresses and is transmitted to healthy corals. The results of these studies will be three-fold: a systematic characterization of nutrient loading and its impacts on reef community structure; characterization of the effects of elevated nutrients on coral disease progression and transmission; and identification of coral species of particular resilience or vulnerability. Results of our research will be disseminated to local government agencies to be used as additional leverage for the need to upgrade sewage treatment on Guam.

B. PROJECT DESCRIPTION

Guam reefs are currently impacted by sewage delivered to coastal waters, through poorly-maintained outfall pipes from two outdated and inefficient primary sewage treatment plants. A plan to *upgrade the current facilities* was recently initiated by local government, after over a decade of increasingly inefficient functioning. However, these upgrades only involve repair of leaks and extension of the outfall pipes. Secondary treatment is planned, but local agencies do not know if, or when, it will be initiated. This problem is of particular urgency given the increase in tourism and plans of the military to relocate up to 20,000 troops and support staff from Okinawa over the next five to eight years. Anthropogenic stress on Guam coral reefs will increase sharply over the next decade as a result of these changes, and is of major concern to local residents and management agencies. *The proposed project will quantify impacts of sewage pollution on coral health and disease, to provide additional evidence of reef degradation to local agencies responsible for water quality.* We will address this issue by quantifying spatial and temporal changes in: 1) coral species diversity and percent cover at increasing distances from sewage outfall sites; 2) seasonal shifts in stable Nitrogen isotopes and water quality at increasing distances from sewage outfall sites; and 3) coral disease progression rate, incidence and severity, at selected sites with varying sewage impacts. N stable isotope analyses of benthic fauna and flora will be used to examine sources and patterns (both spatial and temporal) of nutrient pollution, correlated with shifts in coral community composition and water quality. A controlled nutrient dosing experiment, in which healthy and diseased coral fragments will be exposed to elevated nutrient concentrations in laboratory aquaria, will be carried out to assess the role of nutrient pollution on rates of disease transmission and tissue loss. The results of these complementary studies will be two-fold: a systematic characterization of nutrient loading and its impacts on reef community structure and coral health; and identification of reefs and coral species of particular resilience or vulnerability. Results of this study will be made available to the *Guam Waterworks Authority* (GWA; responsible for sewage treatment), *Guam Environmental Protection Agency* (Guam EPA; responsible for monitoring water quality and identifying compliance violations), and the *Department of Aquatic and Wildlife Resources* (DAWR; responsible for managing coral reef resources and public awareness and education campaigns), anticipated partners in disseminating the results of this study. *Outcomes of this project* will be information transfer to these partners through discussion, a technical report, poster presentation at International Year of the Reef scheduled events, and a manuscript submitted to a peer-reviewed journal. This proposal addresses three of the four goals of the General Coral Reef Conservation Grant program in that it will monitor the *condition of coral reef ecosystems, promote the wise management of coral reef resources,* and *develop sound scientific information on the condition of coral reef ecosystems and the threats to such ecosystems.*

C. NARRATIVE PROJECT DESCRIPTION

i. Identification of Issues:

As in other tropical regions of the world, Guam's coral reefs are subject to increasingly severe anthropogenic impacts. Guam's reefs are used heavily by subsistence and sport fishers, and by the tourism industry for diving and other recreational uses. Guam's economic dependence on tourism and growing population have increased inputs of nutrients, pesticides, and silt in nearshore waters, even as the island remains heavily dependent on its popular dive sites and fishery. Tourism is the second largest industry in Guam, generating US$404.8mil in 2005 alone (www.visitguam.org). In addition, troops, dependents and support staff will be transferred to U.S. military bases in Guam from Okinawa over the next seven years, increasing the current population by 20,000-70,000. The impact on Guam's environment of this population increase is of great concern. Currently, there are three functional sewage treatment plants (Tanguisson, Agaña and Tipalao) which are in compliance with primary treatment standards. The Agaña plant was nonfunctional since the 1980s, and released raw, untreated sewage directly onto the reef until repairs were finally completed in March 2007. The Tipalao plant is under the jurisdiction of the U.S. Navy, and its current condition is not known. Upgrades to Tanguisson and Agaña involve repairing leaks and extending the outfall pipes farther offshore into deeper water, and are scheduled to be completed in 2008 and 2009, respectively (D. Craddick, Guam Waterworks Authority General Manager, pers. comm.). Upgrading to secondary treatment is apparently planned, but no funds have been released and it is not known when this might take place (M. Quezon, Guam EPA, pers. comm.). This impact of chronic nutrient pollution on nearshore coral communities has not been assessed by local resource agencies, though the problem has been identified as one of great concern.

ii. Project Objectives:

The proposed project addresses three priorities of GCRCGP: a) monitoring and assessment of coral reefs, b) coral reef restoration, and c) local action strategies projects. The need for monitoring and assessment of coral reefs is particularly important given the increasing levels of anthropogenic impact on the quality of coastal environments. The use of stable isotope and water chemistry analyses should reveal how current levels of nutrient pollution are impacting coral health, and provide evidence to pressure existing agencies to support planned upgrades. Coral restoration is often best achieved simply by enhancing management, allowing natural recovery processes (regrowth, recruitment) to occur (i.e., passive restoration; Edwards and Gomez 2007). We predict that our study will provide evidence that investments to improve water quality will enhance coral health and reef resilience to future impacts. Given predicted population growth on Guam, a proactive approach to improving water quality prior to this population boom will allow Guam reefs to continue to be an important resource.

In recognition of the growing global problem of disease impacts to coral reefs, and to determine the extend of the problem on Guam reefs, a Local Action Strategy (LAS) for Coral Diseases and Global Climate Change was initiated in 2006 to establish baseline disease prevalence and set up long-term permanent monitoring sites. Surveys have revealed that most diseases recorded elsewhere in the Indo-Pacific (Black Band Disease, growth anomalies, Skeletal Eroding Band, Brown Band Disease, White Syndrome and Ulcerative White Spot Disease; Willis *et al.* 2003; Raymundo *et al.* 2005) are impacting reefs here, and baseline prevalence ranges from 2%

to 14% (Burdick, in prep.). These findings suggest a role of coral disease in the further decline in health of Guam reefs. The LAS has identified an assessment of the role of anthropogenic stressors in coral disease impacts as a major goal. In addition to directly addressing this objective, this proposed project will monitor components of the three priorities of NOAA's national monitoring program: 1) benthic habitat composition, 2) associated biological communities (coral community and disease impacts), and 3) water quality. Outcomes of the project will be directly applied to management, particularly of land-based sources of pollution, and will identify particularly resilient coral species and reef communities which may be recommended for protection.

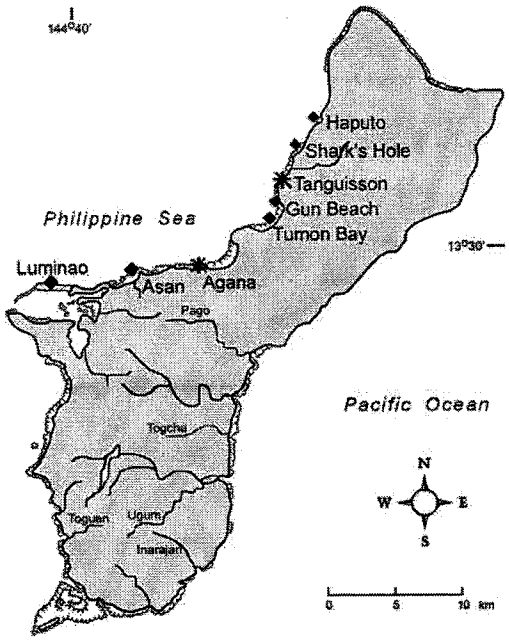

Fig 1. Map of Guam showing proposed sampling sites. Sites with high nutrient inputs (Tanguisson and Agaña) are marked with a ✱; sites with intermediate to low inputs are marked with a ◆ (see Table 1 for additional details)

iii. Project Narrative:

This proposal will test the following hypotheses:

1. Nitrogen stable isotopes reflect nitrogen pollution.
2. Coral diversity and cover are negatively correlated with nutrient pollution.
3. Nutrient pollution increases disease prevalence and severity.

Guam coral reefs provide an appropriate setting for testing these hypotheses. Coral reefs are generally accessible year round and the number of reefs exposed to varying levels of sewage pollution allows for replication of study sites. The University of Guam houses both the Marine Laboratory and the Water and Environmental Resources Institute, which are well-equipped to conduct assessments and long-term monitoring of coral reef health and water quality.

Monitoring sites will be selected at increasing distances from the outfall point sources, along gradients of nutrient inputs. The northwestern coast of Guam is characterized by fringing reefs of similar community structure (robust colonies of *Porites rus,* faviids and pocilloporids, massive *Porites*, and small acroporids), so comparisons between reef patches are possible. A northern sampling gradient will be established with Tanguisson reef representing the

Table 1. Sites selected for monitoring along two nutrient gradients. Sites with ongoing work are indicated with a (●); proposed sites are indicated with a (✓)

Site	Nutrient Inputs	Survey Status	Isotope and Water Sampling	Temperature Monitoring
Tanguisson	high	✓	✓	✓
Agana Bay	high	✓	✓	✓
Shark's Hole	moderate	✓	✓	✓
Haputo	low	✓	✓	✓
Gun Beach	moderate	✓	✓	✓
Tumon Bay	moderate	●	✓	●
Asan	moderate	✓	✓	✓
Luminao	low	●	✓	●

highest nutrient input site, and Shark's Hole and Haputo representing reefs of similar structure but with decreasing nutrient inputs. Sites between Tanguisson and Agaña: Gun Beach and Tumon Bay, represent intermediate levels of input, while sites to the southwest of Agaña Bay: Asan and Luminao represent a decreasing nutrient gradient on the other end of this coastline (Fig. 1). Site selection will be coordinated to compliment on-going monitoring efforts. For instance, Guam Environment Protection Agency (GEPA) monitors water quality at sites around the island that are randomly selected every two years. Thus, no long-term monitoring exists for water quality at permanent sites. Currently, disease prevalence and water temperature are regularly monitored at four sites by L. Raymundo (Table 1). Our project will make use of two of Raymundo's long-term monitoring sites and provide additional information on coral health and water quality on six additional sites.

HYPOTHESIS 1: Nitrogen stable isotopes reflect levels of N pollution.

Nitrogen stable isotope analysis (i.e., $\delta^{15}N$) is becoming a standard tool for understanding coastal nutrient dynamics. This is because $\delta^{15}N$ signatures (a ratio of ^{15}N to ^{14}N, relative to a standard) of plant or animal tissue can reflect the source of N assimilated. For instance, wastewater $\delta^{15}N$ from anthropogenic sources is significantly enriched in ^{15}N due to the loss of "light" ammonia ($^{14}NH_3$) via volatization and conversion of the remaining $^{15}NH_4^+$ to labile nitrate ($^{15}NO_3^-$). Thus terrestrial sources of inorganic N (e.g., NO_3^-), when compared to oceanic sources, may be enriched (i.e., higher ^{15}N content). On the other hand, N from fertilizers is generally "depleted" as there is no fractionation during fixation of N_2. Once in the marine environment, these N sources enter the food web primarily through the uptake of DIN by primary producers and then into consumers via trophic transfer. Thus, stable isotope analysis can be used to detect the presence of sewage-derived or agricultural N in the tissues of primary producers, including autotrophic corals (Heaton 1986). Moreover, because living organisms, like corals and algae, continually "sample" their environment and record it in their tissue or skeletal material, biological materials represent a temporally-integrated proxy for water quality with respect to N pollution. Indeed, a number of recent studies show that both corals and algae can be used to discern sources of N, construct spatial maps of effluent dispersal, and asses the impact of sewage treatment on N loading in coastal environments (Lapointe *et al.* 2004; Costanzo *et al.* 2005; Savage 2005; Ward-Paige *et al.* 2005; Baker *et al.* 2007). Given the proposal to upgrade current primary treatment plants to secondary ones, the data collected as part of this proposal will be invaluable for assessing any ecological impact of upgrading water treatment on Guam coastal marine communities.

Detailed Methodology

For N stable isotope analysis, we will sample both macroalgae and soft corals at each of 8 reefs every 3rd month for 16 months (total of 5 sampling events). From each site, 3 samples of *Caulerpa* sp. (cut 5 cm from tip) and *Sinularia* sp. (cut 5 cm from tip) will be removed. Both of these species are common to the reefs of Guam (Raymundo, pers. obs.) and will allow repeated sampling and comparisons over time and space. During the first trip to Guam, Kim will develop the sampling protocols and assist in collecting the first set of samples. This and subsequent set of samples will be oven-dried for 48 hr at 50 °C and vacuum-packed and stored at -20 °C until all samples have been collected (n = 3 x 2 species x 8 sites x 5 sampling events = 240 samples). This will allow us to carry out all of the stable isotope analyses at one time and complete the work within the 18-month period. Kim's lab will be responsible for preparing the samples for isotope analyses: samples are individually pulverized into power, weighed out to approximately

1.5 mg (± 0.15) for coral tissue, and 3.0 mg (± 0.15) for algae, and placed into 4 x 6 mm tin capsules. All of the samples will be analyzed by the Cornell University Isotope Laboratory (Ithaca, NY). As part of a general site characterization, and to corroborate the isotope analyses, we will also take a water sample at each of the sites monthly using sterile 50 ml centrifuge tubes. The samples will be kept on ice until analysis for nitrate/nitrite, ammonia, and orthophosphate at the Water and Environmental Research Institute of the Western Pacific (8 sites x 1 sample/month x 15 mo = 120 samples). Temperature loggers (HOBO Tidbits®) will be deployed at each site to record water temperature at 15 min intervals for 18 months. This aspect of the work will be conducted by Raymundo.

HYPOTHESIS 2: Species diversity and coral cover are negatively correlated with nutrient pollution.

We predict that sites subject to high nutrient pollution (primarily N) will show lower coral cover and species diversity and greater disease impacts. As corals in high-impact areas have been subjected to nutrient enrichment for an extended time period, we predict that we will be able to detect differences in the coral communities along a gradient of decreasing exposure to sewage nutrient inputs. To test this prediction, we will assess the coral cover, diversity, and disease prevalence and incidence (the number of new disease cases appearing over time) at the eight reefs.

Detailed Methodology

We will conduct initial baseline surveys, and collect samples for isotope analysis, in January 2009. Data already exist for Luminao and Tumon Bay, so additional surveys will be conducted for Agaña Bay, Tanguisson, Gun Beach, Asan, Shark's Hole and Haputo. We will monitor these reefs, representing three levels of nutrient pollution, 3x per year for 15 months, for a total of five survey periods (see Table 1). This will allow us to detect seasonal patterns, if any, in the factors we are monitoring. We will use survey protocols developed by the Coral Disease Working Group of the World Bank Coral Reef Targeted Research Program: three 20 x 2m belt transects at a depth range of 3-5m, where coral cover and diversity are generally the highest. Deeper reef areas around Guam are generally dominated by pavement and coral cover is sparse; we will concentrate our efforts where scleractinian coral is the dominant benthic feature. We will establish equidistant permanent transects parallel to the reef crest (nearshore or slightly beyond the crest, depending on wave energy and accessibility) and note the following: scleractinian coral colony counts (identified to genus), all diseases observed and their host species, and percent cover of: live coral, dead standing coral, coral rubble, pavement/rock, soft coral, macroalgae, sponges, sand/silt. Where coral mortality causation can be determined, it will be noted (i.e., predation, bleaching, disease). These data will be monitored for all sites from which N isotope sampling, water quality and water temperature data are obtained, and statistical correlation analyses (regression, ANOVA) will be conducted to test for significant associations between these parameters and coral species diversity, cover, and disease prevalence. Shifts in the benthic community as a result of changes in water quality will be analyzed using PRIMER. This aspect will be conducted by Raymundo, with Kim participating during visits.

HYPOTHESIS 3: Nutrient pollution increases disease prevalence and severity.

Bruno *et al.* (2003) found that elevated nutrients (as much as 50X greater than ambient) affected the rate at which Yellow Blotch Disease progressed in previously-infected corals, but

did not affect incidence among healthy corals. To further test the effect of nutrients on disease at ecologically meaningful concentrations, we will undertake a set of mesocosm experiments to test the effects of increased N on: 1) progression of White Syndrome lesions (i.e., rate of tissue loss) in diseased fragments; and 2) rate of transmission of White Syndrome to healthy fragments exposed to the disease. As we assume that either susceptible corals have already died out in a site affected by chronic stressors or those that remain are particularly resistant, this experiment will allow us to directly test the effects of chronic nutrient stress.

Detailed Methodology

These experiments will be carried out using branch fragments (i.e., a clonal design) from *Porites cylindrica* collected from Luminao Reef. The branches will be exposed to three levels of nutrients: ambient (water directly from Pago Bay), median (median nitrate levels in Tanguisson) and high (max nitrate concentrations recorded at Tanguisson). A discussion with Guam EPA (M. Quezon) revealed that N and P are not monitored in the wastewater, as primary treatment only necessitates monitoring solids. Therefore, no current data exist from these sites on nutrient concentrations. A previous study by Matson (1991) quantified ambient concentrations from Agaña and Tanguisson outfalls as 1.52 μM and 0.87 μM, respectively for NO_x; and 0.192 μM and 0.196 μM, respectively for reactive PO_4. Tsuda and Grosenbaugh (1977) reported that nitrates in effluents from Agat secondary treatment plant ranged from 10–530 μg/ml (mean = 130 μg/ml). Our dosing experiment is scheduled to take place 10 months after monitoring begins, so we will have nutrient data from three sampling periods prior to setting up the dosing experiment. In September, we will obtain additional water samples from the outfalls using a submerged Van Doren bottle, for analysis of N and P by WERI. The values we obtain from these samples will be used to determine the maximum dosing concentration. A closed system will be used throughout this experiment. Each aquarium will have a separate water supply and aeration, and will be covered to prevent aerosol and spray transfer. Although White Syndrome has been verified in Pago Bay, waste water from aquaria will not be drained into the UOGML waste water system.

To test the effects of elevated N on diseased progression, three fragments from each of five diseased colonies will be collected and randomly assigned to one of three treatments (Fig 2; n = 5 replicates per treatment). The corals will be allowed to acclimate to aquaria conditions for one week after which nitrate levels will be increased with the addition of KNO_3 (made up in seawater; similar volumes of sterile seawater without KNO_3 will be added to the ambient aquaria as a procedural control). Over the course of the experiment, all fragments will be monitored every 3 d for one month for rate of progression of lesions, other signs of ill-health, and colony/fragment fate (i.e., partial or full mortality, or recovery). As handling and measuring can be stressful, measurements and photographs will be taken every three days. Lesions will be examined under a dissecting microscope and photographed weekly to document the increase in surface area of dead skeleton over time using NIH ImageJ. To further characterize an effect of nutrient enrichment, we will carry out a preliminary description of the culturable microbial communities associated with lesions and healthy tissue from a subset of fragments from each treatment. Surface mucous will be aspirated using sterile syringes from lesion margins and

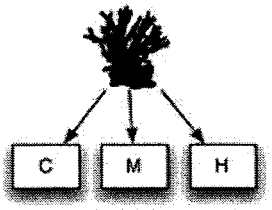

Fig 2. Testing the effects of N availability on corals. Each replicate experiment consists of 3 branch fragments taken from a single colony that are assigned to one of the following treatments: ambient (C), low (L), and high (H) N levels.

healthy tissue, and liquid cultures prepared from these samples. Dilutions will be prepared from these, and plated on several media: Glycerol artificial seawater medium (GASW) is a permissive medium that allows the growth of a wide number of organotrophic bacteria and fungi (Smith and Hayasaka 1982a, b). Eosin methylene blue (EMB) agar will be used as a selective medium for enumerating members of Enterobacteraceae; *E. coli* produces a green sheen on this medium and is indicative of fecal pollution (Gerhardt et al. 1981). Simmons Citrate agar, used for enterics, will be also used to indicate the presence of human waste. Finally, thiosulfate citrate bile salt sucrose (TCBS) will be used to enumerate *Vibrio* spp. Although vibrios are common marine inhabitants, some are also known as coral pathogens. Colony-forming units of all morphologically-distinct forms will be counted and compared across nutrient treatments and between healthy and diseased tissue. At the end of the experiment, we will preserve samples of healthy and diseased fragments for later histopathological examination. This is currently beyond the scope of the present budget, but may be possible in the future with separate funding.

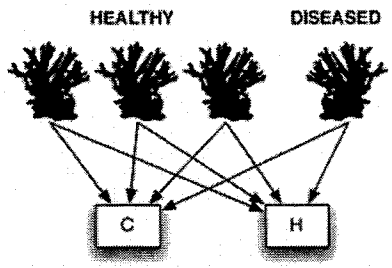

Fig 3. Testing the effects of N availability on disease transmission. Each replicate experiment consists of randomly assigning 3 infected branch fragments from a single diseased colony to each of the 2 treatments. Each treatment will also include 1 branch fragment from each of 3 healthy colonies.

To examine the effects of nutrients on disease transmission, a single diseased fragment will be placed in an aquarium with a fragment from each of three healthy colonies (Fig 3). Each set of fragments (1 diseased + 3 healthy) will be randomly assigned to one of two treatments: ambient and high nutrient levels (Fig 3; n = 10 replicate experiments where each replicate will have a unique set of diseased and healthy colonies from which branch fragments will be taken). As before, fragments will be allowed to acclimate to aquaria conditions for one week prior to the addition of KNO_3 to prescribed levels and monitored every 3 d for one month.

In all aquaria experiments, water will be aerated and temperature maintained at 28 °C - 30 °C. Dissolved oxygen, salinity temperature will be monitored daily using a YSI® meter. To determine frequency of necessary water changes and nitrate monitoring, we will set up replicate aquaria with coral fragments under these culture conditions and monitor nitrate levels daily, using a NitraVer® kit based on a cadmium reduction method (Hach, Loveland CO), prior to setting up the full experiment. Corals will not be fed during this experiment, to avoid rapid deterioration of water quality, and will be fixed into PVC® cups, so direct contact with coral tissue will be prevented. This protocol has been previously used by Raymundo, and healthy fragments appear to respond favorably to these conditions. Water removed during water changes will be transferred into a large vessel to which bleach will be added to a concentration of 10%. After bleach has evaporated, this water will be drained into the soil, away from the water system. Researchers will be gloved, and will change gloves between aquarium censuses. Raymundo has a permit from the DAWR to conduct disease work under these conditions.

iv. Expected Outcomes:

The reefs selected for monitoring in this study are not only those which likely represent varying levels of nutrient input, but are also major sites for diving, fishing and other recreational uses. Our comprehensive approach, testing for correlations between water quality, nitrogen isotopes, benthic community composition, and disease impacts will systematically characterize the

nutrient pollution status on a number of reefs integral to Guam's ecology and economy. The use of nitrogen stable isotopes will allow us to distinguish between fertilizer and sewage pollution. This project is of particular relevance and urgency, as plans for upgrading sewage treatment are on hold, and Guam's population is rapidly increasing. Should our assessments and monitoring reveal an impact of nutrient enrichment, with healthier reefs farther away from high nutrient input, this will provide strong support for a positive effect of improving sewage treatment on Guam reefs.

v. Specific Products:

- A summary technical report put out through University of Guam Marine Laboratory on sewage pollution effects and recommendations relayed to local partners—Guam EPA, GWA, DAWR, National Parks Service and the U.S. Army Corps of Engineers--where the information can be used to strengthen legislation on water quality control and sewage treatment, and to educate the public regarding both impacts of sewage and positive effects of water treatment plant upgrades
- Findings presented at local public awareness events such as Earth Day, UOG Charter Day, and local and regional symposia, via posters and talks
- A manuscript submitted to an international peer-reviewed journal
- Two graduate students (one at UOG and the other at AU) trained in coral monitoring protocols, water quality analyses, and disease diagnosis
- Input for the biannual Report on the Status of U.S. Coral Reefs

vi. Milestones:

This project is scheduled to run for 18 mo. We will first characterize all reef sites with isotope and water quality sampling and deployment of temperature loggers starting in January 2009. Surveys of the all sites will continue every 3 months for 15 months; the last three months will be devoted to data analysis and manuscript and report preparation. Dosing experiments will be carried out in October of 2009 during Kim's second visit. Isotope analyses will be completed during the last two months of the project.

D. NARRATIVE BUDGET SUMMARY

Please see Table 2 for a breakdown of proposed expenses. The proposed budget provides for a part-time graduate student assistant at the University of Guam, who will receive training on underwater disease diagnosis, monitoring, and laboratory protocols for the dosing experiment, and will carry out much of the fieldwork. Summer salary is also requested for a graduate student from American University who will accompany Dr. Kim to Guam. Due to the time needed to carry out field monitoring, sampling and processing of isotope and water samples, and setting up and maintaining laboratory experiments, these student research assistants are needed. The proposed budget and fringe benefits are the standard current rate at the University of Guam and at American University. Other expenses primarily include rental of boats and trucks for transport to the field sites, and SCUBA tanks for dives. Additional expenses include underwater temperature data loggers and funding for communication and shipping.

Travel for Kim and a graduate student is budgeted for a visit at the start of the project to assist in the setting up of both field and laboratory experiments. Kim will make a second trip in October 2009 to assist in the set-up of the dosing experiment (a total of 3 person trips x $1,800

per trip = $5,400). Per diem to cover cost of food and lodging is being requested at a rate of $70 per person (x 42 person days = $2,940). Funds for consumables are being requested at $600 which includes a vacuum sealer and bags, and $300 for shipping and postage. Kim's lab will prep the coral and algae samples prior to sending them to Cornell University's Isotope Lab for N isotope analyses at a rate of $16 per sample (x 260 samples = $4,160 [we are asking for funds to analyze more than the 240 samples that will be collected for the study in case some samples have to be run multiple times]).

Although the University of Guam is exempt from a matching fund requirement, in-kind contributions from the Raymundo lab include: a fully-equipped wet lab for the dosing experiment, two underwater still cameras ($1,000), three sets of SCUBA gear ($2,500), a Wild dissecting scope and a Leica light microscope, equipped with a Jenoptik camera ($18,000), access to a spectrophotometer for nitrate assessment ($6,000), and laboratory bench space and consumable supplies for microbial community characterization ($2,000). Raymundo will commit 10% of her time to this project ($5,400).

Table 2. Proposed Budget for Nutrient & Coral Disease Study

Budget Category	Quantity	$ Cost
Personnel		
UOG part-time graduate research assistant	1 @ 18hr/wk ($12.58/hr X 78 wk)	$17,662
benefits for UOG research assistant	10% of salary for 18mo	$1,766
AU graduate student summer stipend	40 hr/wk x 6 wk	$2,880
benefits for AU research assistant	8% of salary	$230
Travel		
Round-trip airfare, DC–Guam	$1800/trip x 3 person-trips	$5,400
Per diem support in Guam	$70/day X 42 days	$2,940
Rentals		
boat, tank, truck rental	$260/trip x 16 trips	$4,160
Materials and consumable supplies		
sampling supplies, sealer, bags	4 boxes	$600
Stable Isotope Analysis	$16 per sample x 260 samples	$4,160
Water Quality analysis	$30 per sample x 120 samples	$3,600
NitraVer Nitrate test kits	$25 each x 3 kits	$75
Administrative costs		
communicating, mailing, shipping		$300
Indirect costs, UOG	22% of student salary	$3,950
Indirect costs, AU	11.1% Off-Campus standard rate	$1,371
TOTAL COST		**$49,094**

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CURRICULUM VITAE

LAURIE JEANNE H. RAYMUNDO

(i) Professional Preparation

2000 Ph.D. Marine Ecology. Cornell University
1987 M.Sc. Water Resources Management. State University of New York College of Environmental Science and Forestry
1981 B.Sc. Zoology. SUNY College of Environmental Science and Forestry

(ii) Appointments

2004-present: Assistant Professor of Biology, University of Guam
2000-2004: Associate Professor of Biology, Silliman University
1990-2000: Assistant Professor of Biology, Silliman University
1989-2004: Research Associate, Silliman University Marine Laboratory, Dumaguete City, Philippines
1988-1990: Instructor of Biology, Silliman University, Dumaguete City, Philippines

(iii) Grants Awarded

• Co-Principal Investigator, Coral Disease Technical Working Group. Targeted Coral Reef Research Project. Working Group Chair: Dr. C. D. Harvell. Global Environment Facility/World Bank. $140,000 for 4 yr.

• Principal Investigator for coral disease baseline survey and long-monitoring for the coral reefs of Guam. National Oceanographic and Atmospheric Administration. $53,000 for two years.

• Principal Investigator to develop a Coral Disease Working Group for the Central Pacific. NOAA..Co-PIs: A. Bruckner and C. Woodley. $50,000 for one year.

• Co-Principal Investigator with Dr. Alan White. Marine Protected Area Project: Saving Philippine Reefs Through Enhanced Management of Marine Protected Areas in Negros and Cebu, Philippines. NOAA. Funding Approved. $70,000 for two years.

• Principal Investigator for Coral Transplantation Project for Apra Harbor, Guam. U.S. Navy. Funding approved. $60,000 for 18 mo.

(iv) Relevant Publications

Harvell, C.D.H., Jordan, E., Merkel, S.M., **Raymundo, L.J.**, Rosenberg, E., Smith, G.W., Weil, E., and Willis, B.L. 2007. *Coral Disease: Environmental Change and the Balance Between Coral and Microbes.* Oceanography 20(1):172-195.

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(v) Recent Conferences, Workshops, Symposia:

Third International Tropical Marine Ecosystems Management Symposium (ITMEMS 3), Cozumel, Mexico, October 16-21 (Chair, Session on Anthropogenic Impacts to Coral Reefs and Related Ecosystems; Oral Presentation: Marine Protected Areas and Coral Disease: What is the Role of Ecosystem Health in Disease Resistance?)

First Regional Advisory Group Workshop for capacity building within the Coral Reef Targeted Research Project, University of Queensland, Brisbane, Australia, October 10-13, 2005. Representative of the Coral Disease Working Group.

3nd Meeting of the International Coral Reef Initiative General Meeting, Koror, Palau, Oct. 31-Nov. 2, 2005 (Invited Presenter: Coral Disease as an Emergent Management Issue)

CURRICULUM VITAE

KIHO KIM

(i) Professional Preparation

1996 PhD Biology, University at Buffalo
1994 MS Biology, Florida International University
1989 BSc Biology & Environmental Studies, Brock University

(ii) Appointments

2006- Associate Professor, Department of Biology, American University
2003- Director, Environmental Studies Program, American University
2000-2006 Assistant Professor, Department of Biology, American University
1999-2000 Research Associate, Center for the Environment, Cornell University
1996-1999 Postdoctoral Research Associate, Ecology & Evolutionary Biology, Cornell University

(iii) Grants Awarded

2006 NOAA–National Undersea Research Center. The link between coral hosts, surface microbiota and disease ($39,981)
2003 US-EPA, Origins and impacts of the sea fan aspergillosis epizootic explored with molecular and field techniques ($11,693)
2002 NOAA–National Undersea Research Center. Impact of aspergillosis of sea fan corals in the Florida Keys (Rapid Response Support, ~$6,000)
2000 NOAA—National Undersea Research Center, Aspergillosis of sea fans in the Florida Keys: disease resistance and spread (Co-PI with CD Harvell; $20,000)
1999 National Science Foundation, Disease resistance in sea fan corals and host range of the fungal pathogen (Co-PI with CD Harvell; $450,080)
1998 New England BioLabs Foundation, Host-breadth of Aspergillosis (Co-PI with CD Harvell; $30,000)

(iv) Relevant Publications

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EXHIBIT FF

EPA 823-R-07-006

REPORT OF THE EXPERTS SCIENTIFIC WORKSHOP ON CRITICAL RESEARCH NEEDS FOR THE DEVELOPMENT OF NEW OR REVISED RECREATIONAL WATER QUALITY CRITERIA

Airlie Center
Warrenton, Virginia
March 26-30, 2007

U.S. Environmental Protection Agency
Office of Water
Office of Research and Development

June 15, 2007

CHAPTER 2
PATHOGENS, PATHOGEN INDICATORS, AND INDICATORS OF FECAL CONTAMINATION

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2.1 Application of Microbial/Biomarker Parameters

The charge of the Pathogen, Pathogen Indicators, and Indicators of Fecal Contamination workgroup was to identify critical research and science needs in the development of new or revised criteria for recreational waters, including total maximum daily load (TMDL) implementation, and National Pollutant Discharge Elimination System (NPDES) implementation using microbial and chemical indicators. The discussions were limited to constituents for which methods are currently available or expected to be available within the next 3 years and focused around the following four issues:

1. Fecal matter indicators (as surrogates for gastrointestinal [GI] and non-GI illnesses);
2. Pathogens and their index organisms (GI and non-GI illnesses);
3. Application of fecal indicators, pathogen index organisms, and pathogens in combination for criteria development; and
4. Application of all the above for all categories of waters, climatology, and geographical considerations.

Currently, implementation of ambient water quality criteria (AWQC) for the four Clean Water Act (CWA) applications require monitoring fecal bacterial indicators to assess the degree to which the water is contaminated with sewage and sewage-borne pathogens with respect to the accepted risk for exposure. Development of the existing (US EPA, 1986) AWQC for recreational waters were based on epidemiological studies that related concentrations of fecal indicator bacteria at recreational waters impacted primarily by point sources of human sewage.

Since development of the currently used 1986 AWQC, research has shown that this narrow health effects-based standard (i.e., epidemiological studies at beaches with point sources of human sewage) is limited in that it does not take into account differences in geographical conditions, ecology of microorganisms, and varying sources of fecal indicator bacteria. In this regard, the expected relationship between illness and indicator organism densities would be high if the source of contamination is human sewage, moderate if the source was a mixture of human and animal feces, or lower if the source is the result of replication of the indicator bacteria in the environment, such as in soil, sediments, storm drains, or on plants or aquatic vegetative matter. Initially, replication of fecal indicator bacteria was reported in tropical areas (e.g., Hawaii, Guam, Puerto Rico) but has now been documented in subtropical areas such as south Florida and even temperate areas (Great Lakes States). A further but untested complication in interpreting fecal indicator bacteria results may arise due to different rates of pathogen inactivation in the environment relative to fecal indicators across different geographic and climatic regions.

It is for the above reasons that experts in the field of microbial water quality generally agree that the principles of microbial ecology must be considered in water quality assessment. Understanding and applying these principles requires an assessment of the sources of fecal contamination, selection of the appropriate methods used to assess these sources, a connection between the intended AWQC application, and the fecal and/or pathogen indicator or pathogen measured and an analysis of that indicator's fate and transport. Because of this understanding, workgroup members suggested a tiered assessment of a watershed, starting with traditional fecal indicators (conservative measures), and progressing to select a suite of indicators of

contamination (providing source specificity and contaminant load information). A characterization of contaminant inputs through a sanitary investigation of the watershed and the waterbody being assessed should be undertaken, specifically assessing hazardous events, such as rainfall-induced runoff or wastewater treatment failure. Key information would pertain to a cataloguing of point sources (e.g., sewage effluent) and non-point sources (e.g., animals, runoff, on-site septic systems, environmental regrowth) so that a comparative risk assessment can be made based on the concentrations of standard (traditional) monitoring fecal indicators (i.e., *E. coli*, enterococci) and the expected presence of human pathogens. This initial assessment should assist in understanding the relationship between the contamination and epidemiological studies (indicator levels and risks of illness) mentioned elsewhere in these proceedings. In order to select appropriate indicators, a tiered toolbox approach was preferred by workgroup members rather than promoting use of one particular indicator over another.

2.2 Tiered Toolbox Monitoring Approach

An initial cataloguing of fecal pollution sources should include a review of existing monitoring data and a sanitary investigation to assess contaminant levels and sources that impact a given recreational water site. Based on that information, the indicator used in monitoring or the predictive modeling tool most appropriate for each CWA AWQC application and contamination source would be selected for the situation. Water quality assessment for each recreational water site should begin with the simplest analyses and assessment and move on to the most appropriate (specific or targeted) indicator for that site or purpose. More refined tools to differentiate between human, domestic animal, or environmental sources of fecal contamination could subsequently be used if deemed necessary.

If a sanitary investigation determines that fecal pollution is human or animal origin, then *E. coli* or enterococci could be used in the tier one water quality assessment because many pathogens can be expected to multiply in human and animal intestines. If the source of the "fecal" indicator organisms is determined to be from the environment (i.e., from growth in soil/sand, sediment, or water), then *E. coli* and enterococci may be inappropriate because most pathogens are not capable of environmental multiplication. As a result, the monitoring for this tier would need to be a fecal organism/chemical that does not amplify in the environment, such as spores of *Clostridium perfringens* or male-specific (F+) coliphages measured by culture- or molecular-based methods, specific members of the *Bacteriodes* bacteria measured by a molecular method, or use of a chemical indicator of fecal material.

For a subsequent tier of monitoring, infectivity and/or molecular methods could be used for specific groups of pathogens such as, for bacterial pathogens (shiga-toxin producing *E. coli* [STEC] or *Salmonella*), for protozoa (*Cryptosporidium* or *Giardia*), and for representative human sewage-borne viruses (enteroviruses, adenoviruses, polyomaviruses, or noroviruses).

Location-specific data should be archived for potential use in future predictive modeling that might allow for management of site-specific fecal contaminants. Finally, if possible, archiving samples for further characterization and national comparison of new indicators, and/or pathogens and their respective methods would be advantageous assuming a national repository database and sample archive facility could be established.

Several non-GI illnesses have been associated with recreational uses of water but these are not addressed by monitoring for fecal indicator microorganisms or chemicals because the etiological agents for these waterborne diseases come from non-sewage sources. Examples include animal urine (*Leptospira* spp.), shedding from human skin (*Staphylococcus aureus*), or microorganisms that are naturally present in freshwater environments (*Aeromonas hydrophila, Naegleri fowlerii, Legionella pneumophila*). Further, several human pathogenic *Vibrio* species (*V. cholerae, V. vulnificus, V. parahaemolyticus, V. alginolyticus*) are indigenous to marine and brackish waters. Because reliable indicators have not been developed for non-GI etiological agents, the best approach to address aquatic non-enteric pathogens is to characterize the aquatic conditions that increase the risk for these pathogens. For pathogenic *Vibrio* spp., this includes saline waters of warmer temperature and waters that contain high levels of nutrients.

2.3 Parameters for Hazardous Event Pollution Monitoring

The first approach to investigate a hazardous event (sewage discharge, rainfall impact, etc.) would be to assay for fecal indicators (appropriate for a climatic/geographic area of concern, see Section 2.4). The primary indicators of fecal contamination are *E. coli* or enterococci; however, based on the classification from an initial sanitary investigation, alternatives may include *Clostridium perfringens* or F+ coliphages (dependent on a robust method being confirmed). These must be demonstrated to relate to a possible health outcome (see Section 2.4). When information is required on source characterization, then additional microbial indicators (see Section 2.5) are generally preferred over chemical biomarkers (see Section 2.7).

Focused sampling during and after higher risk periods is important when information from the sanitary investigation (which may include system models) is used to predict such risk. For these applications, the context of the likely pathogen group(s) should dictate the type of indicator to assay. For example, for rainfall in an area possibly impacted by on-site septic systems, viruses are considered the most mobile pathogen group so use of virus model organisms, such as the F+ coliphages, would be informative. For sites contaminated by concentrated animal feeding operations (CAFOs), reasonable pathogen index tests include shiga-toxin producing *E. coli* or *Cryptosporidium*. This approach assumes that some background level of the targeted group is known for the area of concern (see Section 2.8, research needs).

2.3.1 Microbiological Parameters

The Beaches Environmental Assessment and Coastal Health (BEACH) Act of 2000 requires States with coastal or Great Lakes recreational waters to adopt the current (US EPA, 1986) criteria for *E. coli* and enterococci. In November 2004, EPA promulgated a final rule that put federal standards in place for the 21 coastal states that had not adopted the 1986 criteria or established criteria as protective of human health as EPA's 1986 criteria. However, these federal criteria apply only to coastal states and Great Lakes waters. In many cases, a fecal coliform-based standard still applies to many states having only inland waters. It is important to note that the results of the epidemiological studies used to generate the 1986 criteria for coastal and Great Lakes waters may not be directly applicable to all inland waters.

2.4 Traditional Fecal Indicators (Coliforms and Enterococci)

Consideration of the environmental context for which these traditional indicators are used is critical to the interpretation of the results. For example, wastewater treatment/disinfection may be effective in reducing the number of these traditional fecal indicators but ineffective in reducing/inactivating some pathogens of concern (Blatchley et al., 2007). Some industrial treatment systems may contain/enable replication of high number of "fecal indicators," which are not necessarily associated with fecal sources (Degnan, 2007; Gauthier and Archibald, 2001). Ambient water and soils in tropical environments may be conducive to the growth of environmental strains of *E. coli* and enterococci (Fujioka and Byappanahalli, 2001). A similar situation was found in temperate Australian waters (Ashbolt et al., 1997; Barnes and Gordon, 2004; Davies et al., 1995) and these indicators have also been found to persist in U.S. beach sand/sediments (Whitman et al., 2006).

The range of strains of fecal indicators identified by traditional culture-based methods may differ from those identified by enzyme-based and quantitative polymerase chain reaction (qPCR [molecular])-based methods. Further, the strains more associated with fecal matter are not differentiated from the environmental strains by all of these methods. There are commercially available systems that can aid in the discrimination of strains; however, less expensive typing kits do not accurately provide such discrimination for environmental strains. It is important to note then when quantifying fecal indicator organisms, different methods target different strains. For example, cells stressed by wastewater disinfection processes may be enumerated using MPN (Most Probable Number) methods but excluded by methods enumerated by colony forming unit (cfu) methods. When current qPCR methods are used, both viable and non-viable cells are detected. In addition, the number of gene targets may vary per cell and therefore do not provide comparable information to culture-based results.

E. coli

Of the traditional fecal indicators (see also Appendix E, Text Box E-1), only *E. coli* has been shown in epidemiological studies to consistently relate to health outcomes for freshwater recreational water users (Cabelli et al., 1982; Wade et al., 2003; Wiedenmann et al., 2006). In marine/estuarine waters, *E. coli* is more readily inactivated than enterococci and appears to correlate less well to health risk than enterococci for saline water environments.

Subtyping of different strains of *E. coli* (library-dependent microbial source tracking methods) appears to be very site-specific if useful at all. Thus, it is not generally suggested as an effective way forward to separate environmental sources of *E. coli* from fecal sources across the United States (see Section 2.5).

Enterococci

The enterococci are the major group of fecal indicators that have a clear link to GI illness and upper respiratory disease in bathers in marine and fresh recreational waters (Kay et al., 2004).

There are, however, several shortcomings in the use of current methods for enterococci. Most importantly, there is a range of different *Enterococcus* spp. detected by current methods. Based on unpublished Californian studies (Stephen Weisberg, SCCWRP, personal communication, 2007), greater fecal specificity may result from specific identification and enumeration of *E. faecalis* or *E. faecium* or molecular-based methods targeting specific genes within these species (e.g., ribosomal RNA or enterococcal cell surface-associated protein and its gene *Esp*) (Lehner et al., 2005; Liu et al., 2006). However, no robust method is currently available that readily provides such information, nor has this concept been verified at other U.S. recreational water sites (Anderson et al., 1997).

2.5 Alternative Fecal Indicators

2.5.1 Bacteria

Clostridium perfringens

C. perfringens is a member of the sulphite-reducing clostridia (SRC), which are spore-forming anaerobic bacteria excreted in human and animal fecal matter, but unlike other SRC, do not appear to grow in the aquatic/soil environment. These bacteria have been used as fecal indicator organisms for decades. Australian and North Carolina studies show *C. perfringens* levels in humans comparable to levels found in dog and feral pig feces, but low levels in cattle, sheep, horses, and birds (Leeming et al., 1998; Mark Sobsey, University of North Carolina, Chapel Hill, personal communication, 2007). Importantly, because *C. perfringens* does not appear to grow in aquatic/soil environments, it has potential to be useful as a fecal indicator for tropical environments such as in Hawaii where growth of *E. coli* and enterococci in soil/sand, sediment, and water make those indicator organisms less useful (Byappanahalli and Fujioka, 1998; Hardina and Fujioka, 1991; Roll and Fujioka, 1997). For example, in ambient streams in Hawaii, concentrations of fecal coliforms, *E. coli*, and enterococci consistently exceed recreational Water Quality Standards due to contribution by extra enteric sources (Hardina and Fujioka, 1991, Luther and Fujioka, 2004). Thus, monitoring inland and coastal waters for *C. perfringens* provides reliable data for sewage contamination and is used by the Hawaii State Department of Health to confirm a sewage contamination event (Fujioka and Byappanahalli, 2001).

The presence of *C. perfringens* (spores) in water, therefore provides evidence of existing human/urban fecal contamination, which may reflect either recent or historical fecal contamination from humans or animals. Although methods have been available for some time, confirmation of a robust and consistent method approach should be developed. For example, the advantages of heat-treating samples (or not) to remove background vegetative cells and induce spore germination remains unclear.

The environmental resistance of *C. perfringens* spores has both advantages and disadvantages in their application as a fecal indicator, pathogen indicator, and as an indicator of wastewater treatment efficacy. Collectively, these make *C. perfringens* spores better indicators of persistent and treatment-resistant pathogens, such as *Cryptosporidium* oocysts (resistance to chlorine) and adenoviruses (resistant to UV radiation). However, they can be so persistent in the environment

that they may not indicate the presence of pathogens coming from recent (contemporary) fecal contamination.

Recent studies on the partitioning of *C. perfringens* and other fecal indicator microbes in environmental waters, such as *E. coli* and coliphages, indicate differences in the extent of their association with settleable particulate matter (Characklis et al., 2005; Krometis et al., 2007). To date, limited data have been collected on any potential relationship between *C. perfringens* counts and recreator health outcomes (see Section 2.8).

Bacteroides

Bacteroides spp. are members of the normal microbiota of warm blooded animals and studies have shown them to be among the most prevalent genera in feces (Holdeman et al., 1976). Because they are strict anaerobes that grow in the GI tract of humans and animals, they do not survive for long periods of time under aerobic conditions (Kreader et al., 1998). However, their survival under different redox potential conditions (e.g., sediments) has not been thoroughly studied. Recent research based on molecular methods has demonstrated that some isolates may be strictly associated with human feces (Walters et al., 2007). If this is the case, these microorganisms also have the potential to be used for microbial source tracking (MST) applications.

Studies have indicated human versus bovine specificity in certain 16S rRNA genes therefore, 16S rRNA *Bacteroides* genes have been used as an index of human or animal contamination in Europe and the United States. The ability to differentiate sources of fecal contamination is very attractive when it comes to determining risk as a result of exposure via recreational waters. The molecular methodology has been shown to be robust and applicable in the United States and Europe, though it remains to be seen if this robustness holds across temperate versus tropical or subtropical zones of the world. Some results from Hawaii and Europe indicate that these methods may be useful under those climatic conditions (Betancourt and Fujioka, 2006; Seurinck et al., 2006). Either way, it is unclear whether quantification of human/animal fecal loads will be consistent or indeed possible using these molecular-based methods.

Though data from molecular techniques have shown that there is specificity in the human versus animal strains, the fact that both human and animal feces contain a diverse population of *Bacteroides* spp. may limit the usefulness of some detection methods. Methods that focus on one target may have reduced sensitivity as a result of the lower concentrations of a specific *Bacteroides* strain. Data have shown that *Bacteroides* spp. does not survive for long periods of time in the environment; thus, *Bacteroides* detected by qPCR in ambient waters includes a high percentage of inactivated microorganisms. The fact that qPCR detects both live and dead organisms needs to be considered when data are applied in different contexts (e.g., different AWQC applications). That is, qPCR detection is linked to the time the nucleic acid remains within the cell without being degraded. EPA data have demonstrated that the DNA remains undegraded for up to 20 days (Kevin Oshima, USEPA, Office of Research and Development, personal communication, 2007) in the inactivated unlysed cells. This may be equivalent to the survival of some enteric pathogens under environmental conditions. Thus, the presence of *Bacteroides* may have possible use as an indicator of health effects. Because the concentration

of *Bacteroides* spp. in feces is much higher than other fecal bacteria, once the persistence of PCR-detected types is better understood, it may also be useful for TMDL applications, although this possibility needs further evaluation.

The molecular methodology for the detection of general and human-specific *Bacteroides* spp. is already being tested and has proven to be robust (Gawler et al., 2007; Walters et al., 2007). Thus, if detection methods are validated in the United States there is an excellent opportunity for short-term advances in quickly adapting the use of this alternate indicator for the rapid analyses of recreational waters and fecal source identification.

2.5.2 Bacteriophages

Coliphages

Bacteriophages (viruses) that infect *E. coli* and possibly other closely related coliform bacteria are called coliphages. There is a long history of research documenting the possible uses of phages as indicators of fecal contamination (Grabow et al., 1998). Coliphages were first proposed as indicators of the presence of *E. coli* bacteria and are taxonomically very diverse, covering the following six virus families: three families of double-stranded DNA viruses (*Myoviridae*, *Styloviriae*, *Podoviridae*), two families of single-stranded DNA phages (*Microviridae* and *Inoviridae*), and one family of single-stranded RNA viruses (*Leviviridae*).

Coliphages that infect via the host cell wall of *E. coli* are called somatic coliphages (including families *Myoviridae*, *Styloviriae*, *Podoviridae*, and *Microviridae*). Male-specific (also called F+) coliphages (*Inoviridae* and *Leviviridae*) infect by attaching to hair-like appendages called F-pili protruding from the host bacterium surface.

Somatic phages have been explored as fecal, treatment efficacy, and health effects indicators. However, little is known about the specificity of their occurrence in human or animal feces. Furthermore, their considerable taxonomic diversity and the lack of readily available and convenient methods to distinguish or specifically detect the different groups has made it difficult to determine which, if any, are effective fecal, treatment efficacy, or health effects indicators. In a recent study by Colford et al. (2007), somatic coliphages were not predictive of human health risks from bathing in marine recreational water largely impacted by non-point sources of fecal contamination. Furthermore, there is very little information on the sources and ecology of the somatic coliphages, especially for the different taxonomic groups. With rare exceptions, they are detected as a broad group with no effort to identify specific taxonomic groups or relate or attribute these different taxonomic groups to specific sources of human or animal fecal contamination or possibly non-fecal environmental sources.

Male-specific coliphages have been studied extensively as fecal indicators and for water/wastewater treatment/disinfection efficacy. Furthermore, F+ RNA coliphages can be distinguished genetically (via nucleic acid detection methods) or antigenically (via immunological methods), into four distinct subgroups: I, II, III, and IV. There is reasonably good evidence that Groups II and III are associated primarily with human fecal waste and that Groups I and IV are associated primarily with animal fecal waste (Furuse et al., 1975; Hsu et al.,

1995; Osawa et al., 1981) in the United States. Male-specific coliphages have been included in some epidemiological studies of recreational water. In the recent study by Colford et al. (2007) at a marine recreational water site impacted primarily by non-point source fecal contamination, F+ coliphages were the only microbial indicator whose levels were associated with risks of swimming-associated illness.

Strengths of Coliphages as Indicators

Advantages of both somatic and F+ coliphages as fecal indicators include their (1) presence in relatively high concentrations in sewage; (2) relatively high persistence through wastewater treatment plants, compared to typical bacterial indicators like *E. coli* and fecal coliforms (coliphages may behave similarly to human viruses during wastewater treatment); and (3) ability to be detected in relatively small (100 mL) to medium (1,000 mL) volumes of fecally contaminated water.

Coliphages can be detected by relatively simple, affordable, and robust culture methods—several of which have been standardized and collaboratively tested as EPA, EU, and ISO (International Organization for Standardization) water methods. However, the EPA methods for somatic and F+ coliphages have been fully validated only for groundwater and not for ambient surface waters or wastewaters. Recent research also describes a rapid, simple, and affordable method to detect and group infectious F+ coliphages by short-term (3-hour) enrichment culture, followed by quick (<1 minute) detection of positive cultures by a simple immunological (particle agglutination) method scored by simple visual examination (Love and Sobsey, 2007). The method can be conducted in an MPN format to quantify concentrations of the different F+ coliphage groups (F+ DNA and F+ RNA Groups I, II, III, and IV).

These findings indicate that robust, simple, rapid, and low-cost F+ coliphage methods could be implemented within the 2 to 3 year time frame if correlations to health targets are observed in epidemiological studies. It would be valuable if water samples from upcoming EPA and SCCWRP (Southern California Coastal Water Research Project; see also Appendix F) marine recreational water epidemiological studies are collected and archived for analysis by these emerging qPCR methods once they are fully developed and validated. In addition, research is suggested to compare the performance of methods for rapid coliphage detection by short-term enrichment-particle agglutination and qPCR and to consider the advantages and disadvantages of these two methods for application to recreational water quality monitoring.

Limitations of Coliphages as Indictors

Although effective methods are available to recover, detect, and quantify coliphages, limitations and unsolved problems with these methods remain. The single agar layer method (EPA Method 1601) for enumeration of coliphages by counting plaques is limited to sample volumes of about 100 mL. Analyzing larger volumes is cumbersome and consumes considerable materials, such as Petri plates. Although the enrichment culture-spot plate method can be used to conveniently analyze sample volumes of up to 1 L, the method makes it more difficult to resolve coliphage mixtures when more than one type of coliphage is present in the enriched sample volume. In some cases, one coliphage will grow faster and to a higher concentration. This makes it difficult

to detect and isolate minority coliphages that grow more slowly and to lower concentrations. However, detection of all of the different coliphages present as a mixture in enriched sample is possible by either nucleic acid or immunological (particle immunoagglutination) methods.

The ecology of both somatic and F+ coliphages remains poorly documented and inadequately understood. Information is lacking on bacterial host range, sources, occurrence, and behavior (survival, transport, and fate) in different geographical regions having different climates (temperate, subtropical, and tropical) and in waters and wastewaters of different microbial quality.

F+ coliphages can also be detected by molecular-based methods, including conventional and qPCR methods, according to recent studies. Careful review of these studies suggests that there may be deficiencies in the ability of these qPCR methods to detect the broad range of F+ DNA and F+ RNA coliphages and their subgroups. Nevertheless, research is now in progress to further improve F+ RNA qPCR by developing and performance-validating primer sets for all four genogroups of F+ RNA coliphages (Stephanie Friedman, EPA Environmental Effects Research Laboratory Laboratory, personal communication, 2007). Reliable methods have not been developed for genetic analysis and characterization of different somatic coliphage taxonomic groups.

Very few studies have been conducted to evaluate F+ coliphages as predictive indicators of human health risks from recreational use of water. The most extensive study was conducted by Colford et al. (2007). That study showed no health relationship for somatic coliphages, but a weak relationship for F+ coliphages examined by two different assay methods—an MPN version of EPA Method 1601 (enrichment-spot plate method) and EPA Method 1602 (saline agar layer plaque assay). However, these methods have not been performance characterized and fully validated for use in fresh and marine recreational waters according to EPA collaborative study protocols. Additional studies of this type are needed to clarify their potential criteria uses.

Bacteroides phages

Bacteroides phages, viruses that specifically infect *Bacteroides* spp., have been tested as indicators of fecal material in Spain and more recently in the U.K. The former used a method (bacterial host) that was tested in some labs in the United States but further efforts were not made as a result of the perceived difficulty in dealing with anaerobic methodology. Attempts to use the *B. fragilis* strain VPI 3625 showed low occurrence of these phages in the United States (Chung and Sobsey, 1993). Spanish data initially supported the use of *B. fragilis* HSP40, which is specific to phages that only occur in human feces. More recent British work indicated human specificity and high phage counts for a newer Spanish host *Bacteroides* (GB-124), thus providing the opportunity for determining human fecal contamination and virus transport using a rapid and inexpensive phage method (Ebdon et al., 2007).

Strengths of Bacteroides Phages as Indicators

The methods for the detection of *Bacteroides* spp. phages are inexpensive and their presence indicates human fecal contamination. In addition, there is research that indicates specific

Bacteroides hosts are susceptible to phages that are possibly useful for MST, which would be beneficial for its use for CWA §304(a) criteria (Chung and Sobsey, 1993; Ebdon et al., 2007).

Limitations of Bacteroides Phages as Indicators

The diversity of phages including their specificity for human host strains is not yet well characterized over a range of locations. This type of data could be easily obtained in 2 to 3 years, but if it is discovered that there is wide variability in their validity for MST, then their attractiveness for use in national AWQC would be reduced. Many laboratory personnel may not have the experience required to work with anaerobic microorganisms; however, little additional laboratory equipment would be required. Because detection methods have not been standardized in the United States, it would likely take several years to develop standardized methods for enumeration of *Bacteroides* phages in water samples.

2.5.3 EU Project Summary of Tracers

Several microbes and chemicals have been considered as potential tracers to identify fecal sources in the environment. However, to date, no single approach has been shown to accurately identify the origins of fecal pollution in all aquatic environments. In a European multi-laboratory study, different microbial and chemical indicators were analyzed in order to distinguish human fecal sources from nonhuman fecal sources using wastewaters and slurries from diverse geographical areas across Europe. Twenty-six parameters, which were later combined to form derived variables for statistical analyses, were obtained by performing methods that were achievable in all the participant laboratories and include the following: enumeration of fecal coliform bacteria, enterococci, clostridia, somatic coliphages, F+ RNA phages, bacteriophages infecting *Bacteroides fragilis* RYC2056 and *Bacteroides thetaiotaomicron* GA17, and total and sorbitol-fermenting bifidobacteria; genotyping of F+ RNA phages; biochemical phenotyping of fecal coliform bacteria and enterococci using miniaturized tests; specific detection of *Bifidobacterium adolescentis* and *Bifidobacterium dentium*; and measurement of four fecal sterols. A number of potentially useful source indicators were detected (bacteriophages infecting *B. thetaiotaomicron*, certain genotypes of F+ bacteriophages, sorbitol-fermenting bifidobacteria, 24-ethylcoprostanol, and epicoprostanol), although no one source identifier alone provided 100% correct classification of the fecal source. Subsequently, 38 variables (both single and derived) were defined from the measured microbial and chemical parameters in order to find the best subset of variables to develop predictive models using the lowest possible number of measured parameters. To this end, several statistical or machine learning methods were evaluated and provided two successful predictive models based on just two variables that provided 100% correct classification—(1) the ratio of the densities of somatic coliphages, and phages infecting *Bacteroides thetaiotaomicron* to the density of somatic coliphages and (2) the ratio of the densities of fecal coliform bacteria and phages infecting *B. thetaiotaomicron* to the density of fecal coliform bacteria. Other models with high rates of correct classification were developed but they required higher numbers of variables (Blanch et al., 2006).

2.6 Pathogens and Pathogen Indicators

Many beach regulators and scientists believe that there are significant opportunities to utilize specific pathogens or pathogen indices to better understand or characterize potential health risks from recreational exposures. Some reasons for not doing so, however, remain—especially that pathogen numbers are generally significantly lower and more variable than fecal indicator organisms. Nonetheless, pathogens could be utilized to accurately determine risks as there have been a number of studies that define actual human dose-response from oral exposures such as may be encountered during swimming. Enteric pathogens are found in raw and even treated sewage so there is merit in using them in water quality monitoring to assess the risks from exposure. Also, it is possible that an entire "class" of pathogen risks can be determined by the presence of an "index pathogen" representing that group. The current capabilities of molecular methods to detect, identify, and enumerate pathogens has increased regulators' and stakeholders' interest in seeing these applied to ambient water quality monitoring to better protect public health.

There are a number of criteria related capabilities that may be provided by use of specific pathogen or index pathogen monitoring, such as the following: (1) determination of specific pathogen residuals from sewage discharges, the data from which could then be used to conduct quantitative microbial risk assessment (QMRA) studies to assess relative levels of public health concern at a beach; (2) establishment of "model" pathogens and index pathogens that could be used to assess risks from new or reemerging pathogens (an example would be the use of a virus model to assess the recreational risks from avian influenza [H5N1] because this virus can be released from infected human and animal feces [especially waterfowl] and can directly or indirectly contaminate recreational waters); and (3) determination of levels of pathogens that can subsequently be used in QMRA studies to inform decision making relative to whether or not a beach should be closed or reopened after a closure.

There are currently two approaches to pathogen detection, identification, and enumeration, (1) the traditional culture-based techniques that are especially useful in determining viability of the sampled materials; and (2) the molecular-based methods (PCR, antibody-based, and metabolic-based) that generally cannot distinguish between viable and non-viable pathogens, but which may be quite useful in further differentiating or speciating pathogens in water samples. The culture-based methods are useful for recreational waters in that they can determine if there is a viable disease risk from exposure while the molecular methods may not be capable of discerning viability.

Moreover, the culturable isolate can be further characterized for the presence of human virulence genes and compared to clinical isolates in waterborne disease outbreaks. In contrast, molecular-based methods may not be capable of discerning viability although the presence of virulence genes can also be assayed by molecular methods. Because molecular methods do not recover the entire microorganism, further characterization of that microorganism is limited.

Specific tracking of host sources using molecular techniques for pathogens can be very useful in setting TMDLs, as it can help identify the source of the pathogen and its magnitude. Recent improvements in molecular science applications have brought about a capability to

simultaneously sample and evaluate large numbers of pathogens (e.g., microarray technology). Microarray technology still requires high concentrations of pathogens for detection. However, ambient waters generally contain pathogen levels below the limits of detection and are unevenly distributed in the water matrix. Thus, research is needed to determine how to best apply these advanced technologies for characterizing enteric and non-enteric disease contaminants, their levels, and potential risks associated with their presence in recreational waters.

Workgroup members expressed some concerns about using either specific pathogens or pathogen class indices as a first tier monitoring requirement for infectious disease risks in a recreational water setting. First, pathogens are typically present in low concentrations in treated sewage, receiving waters, and also in recreational waters; therefore, high volumes of water need to be sampled, which is time consuming, costly, and contributes to analytical variability. Second, pathogen presence is typically sporadic in a community as many waterborne diseases may not be endemic, but are rather transient/episodic so they do not represent a constant contaminant source of fecal pathogens to monitor. Third, there is a variable component in terms of fecal contributions from humans and various animal sources in ambient waters that may have an impact on determining recreational exposure risks. Typically, a number of the bacterial pathogens (e.g., toxigenic *E. coli*, *Campylobacter*) are found in both humans and animals, but there may be differences in strain virulence or infectivity potential from different sources. Likewise, there are a number of protozoan pathogens that cross-infect animal species and humans (*Giardia* spp. and *Cryptosporidium parvum*). On the other hand, human enteric viruses have a much more limited host range and except for a potential few (e.g., hepatitis E virus [HEV]), animal sources of enteric viruses are not a major public health concern in recreational waters. Lastly, it is important to note that at any given time only a small portion of the human population may be infected and excreting any specific pathogen or index pathogen. Thus, large wastewater treatment systems may always contribute a small level of pathogens of concern while septic systems or small treatment systems may not have enough contribution from the infected population to ensure that those effluents would contain specific pathogens of concern to use as a routine measure of contamination—even if the disease organisms are endemic in the population. Also, many types of pathogens are associated with a seasonality or periodicity to their occurrence in a given population.

It is reasonable to use specific pathogens or their index organisms (or model organisms) in a toolbox or tiered approach to monitoring if considered as other than as a first tier measure of fecal contamination. In a toolbox approach, the determination of the presence and concentration of specific pathogens or their index organisms could be useful to characterize risks once it has been determined that there is a trigger level of fecal contamination at a given recreational water site. Dose-response data for a number of the primary pathogens from oral exposures is available and these data would help more narrowly define exposure risks for a detected pathogen. Because of the costs, time for analysis results, and expertise needed to test specific pathogens or index organisms, these measurements would be the last set of measurements applied to monitoring of recreational sites for determining potential sources. The specific pathogen monitoring tools for other AWQC applications (e.g., TMDLs) could allow States to determine sources and concentrations of the pathogens for particular upstream contamination events. Also, pathogens could be incorporated into future NPDES permit limits and be used in the future to assess

wastewater treatment plant discharges for specific pathogens of concern downstream and to provide a better understanding of the efficacy of treatment and disinfection processes.

2.7 Chemical Biomarkers of Fecal Contamination

Various shortcomings have been identified in relying solely on indicator bacteria or pathogen/index microorganisms for CWA criteria uses. Methods for MST in aquatic environments have been developed and discussed above that distinguish animal from human sources in the United States and in Europe (Blanch et al., 2006). However, for some specific tiered approaches in sanitary investigations, certain chemical biomarkers of sources may provide timely or higher resolution information in fecal source tracking. Some of the most promising are discussed below.

2.7.1 Fecal Sterols

The most commonly known fecal sterol, coprostanol (5β-cholestan-3β-ol), is largely produced in the digestive tract of humans and dogs by microbial hydrogenation of cholesterol (Leeming et al., 1996). The term "sterols" is generally used for all sterols and stanols (i.e., "fecal sterols") and is also a more specific term denoting a steroidal alcohol with at least some degree of unsaturation.

Two pathways have been proposed for the biotransformation of cholesterol to coprostanol, one in the gut and the other in natural sediments. The α-configured form (cholestanol) is the most thermodynamically stable of the reduction products and is found ubiquitously in the environment; whereas coprostanol is largely of fecal origin, but some reisomerization can yield low levels in natural sediments. Both forms are easily resolved by gas chromatography-mass spectrometry (GC/MS) analysis.

An important advance in using these fecal sterols has been the realization that it is critical to measure both the ratios and absolute concentration of at least four of these related compounds to attribute fecal source contributions between humans, herbivores, and birds (Ashbolt and Roser, 2003). Coprostanol alone has never really been embraced as an indicator for sewage pollution because its presence is not considered as indicative of a health risk due to multiple sources and low level environmental production in sediments.

The fecal sterol biomarker technique offers many diagnostic and quantitative advantages when used in conjunction with traditional techniques for detecting sewage pollution. When careful data interpretation is undertaken, fecal sterol analysis, although expensive and complex, has resolved problems of source attribution in urban and rural environments not possible with use of traditional fecal indicator bacteria and coliphage assays (Roser and Ashbolt, 2007).

2.7.2 Caffeine

Caffeine has been extensively examined as a tool for assessing human influence on aquatic systems. Although caffeine is metabolized when consumed, a small amount (<10%) of ingested caffeine remains intact when excreted (Peeler et al., 2006). Most work in the past decade has

focused on heavily polluted systems and efficiency of caffeine removal in sewage treatment plants, although with improvements in techniques and the lowered detection limits, the scope of application has broadened to include stream, wetland, estuarine, and groundwater systems.

A major disadvantage is that caffeine is often present in the urban environment from numerous plant species debris as well as from human "dumping" of coffee wastes. Further, the current methods used (specific extraction and GC/MS analysis) are relatively complex and expensive. Nonetheless, based on the recent work of Peeler et al. (2006) in southwest Georgia, caffeine appears immediately below wastewater discharge sites and within towns, but not in rural watersheds. Overall, aquatic concentrations of caffeine are typically less than for fecal sterols, but caffeine tends to stay in solution, whereas the sterols associate with fine particulates.

2.7.3 Optical Brighteners and Other Sewage Markers

Recent sewage contamination may be readily identified in waters by the presence of ammonium, turbidity/particle counts, phosphate, odor, and a range of organics present. Depending on the sensitivity and AWQC applications, some of these analytes may provide value in fecal source identification.

One relatively inexpensive and sensitive fecal source identification method is fluorometry (Hartel et al., 2007). Fluorometry identifies human fecal contamination by detecting optical brighteners (also called fluorescent whitening agents) in water. Optical brighteners are compounds added primarily to laundry detergents, and because these brighteners emit light in the blue range (415 to 445 nm), they compensate for undesirable yellowing in clothes (Kaschig, 2003). In the United States, 97% of laundry detergents contain optical brighteners (Hagedorn et al., 2005). Because household plumbing systems mix effluent from washing machines and toilets together, optical brighteners are associated with human sewage in septic systems and wastewater treatment plants. However, in order to use optical brighteners to detect human fecal contamination properly, they must be combined with use (counts) of fecal indicator bacteria. For example, effluent from a wastewater treatment plant contains optical brighteners, regardless of how effective the treatment processes have been at removing or inactivating pathogens. Thus, data on the presence of optical brighteners without accompanying data on viable fecal indicators does not provide information on the potential health risk from pathogens.

However, results of studies that have combined fluorometry with counts of fecal bacteria have been contradictory. Although various reports have documented a strong fluorescent signal and high numbers of fecal enterococci, cases of no correlation between fluorometry and counts of fecal bacteria have also been reported (Hartel et al., 2007). One key confounder has been the presence of organic matter that fluoresces and interferes with fluorometry. Yet, this interference can be reduced by adding a 436-nm emission filter to the fluorometer, which may reduce background fluorescence by over 50%. As long as the fluorometer used is equipped with a 436-nm filter, it appears that targeted fecal indicator sampling combined with fluorometry can be a relatively inexpensive method for identifying human fecal contamination in water.

In summary, chemical biomarkers appear to have niche applicability for those with the resources and expertise to use them and where such biomarkers are advantageous, such as where other less expensive MST options have shown to be unsatisfactory or provide ambiguous results.

2.8 Research Needs

2.8.1 Near-term (1 to 3 Years)

1. Validate the range and species or sub-species diversity qPCR assays identify, and how they may relate to health outcomes for recreational exposures (also using archived epidemiological study material) (**high priority**).
    a. Example priority list of organisms: enterococci, *Bacteroides*, *C. perfringens*, *E. coli*, F+ RNA coliphages, and somatic coliphages
2. Investigate the potential for speciation of enterococci to identify fecal-specific (preferably human) from environmental strains, then apply results to future MST and epidemiological studies (**high/medium priority**).
3. Ensure that archived samples (collected from epidemiological/specific studies) are suitably sorted and stored (to maintain their integrity) for future viability as well as molecular-based method comparison or validation studies for candidate indicators/methods (**high priority**).
4. Validate *C. perfringens* (SRC) assay's robustness over a range of water and sediment sample characteristics and correlate health effects relationships to this indicator (**high priority**).
5. Determine if there are *Bacteroides* analytical targets that are human-specific and validate their use over a range of geographic areas, diverse populations, climates, and water quality conditions to correlate levels to health targets (**high priority**).
6. Conduct health and epidemiological studies with as wide a range of microorganisms (indicators/MST organisms) as possible to identify risk correlations for a range of pathogens/indicators (including bacteriophages) from various nonhuman sources; at a minimum would include *E. coli*, enterococci, enterococci-qPCR, coliphages, *Bacteroides*-PCR, *C. perfringens*; where possible, *Bacteroides* phage GB-124, enterohemorrhagic *E. coli* (EHEC); and check for absence of human Norovirus-qPCR, adenovirus-qPCR, Pan-enterovirus-qPCR, polyoma viruses (**high priority**).
7. Conduct health and epidemiological studies with microorganisms from nonhuman sources such as *Leptospira* spp. in fresh and *S. aureus* and pathogenic *Vibrio* spp. in marine recreational waters and determine appropriate indicators for these pathogens (**medium priority**).
8. Conduct epidemiological studies incorporating the measurement of pathogens of interest (along with indicators) as monitoring tools in sewage in order to determine the correlations of the occurrence of these pathogens to indicators, and to better understand their association with diseases at downstream recreational locations. For instance, while it is strongly suggested that enteric viruses are major contributors to illness from swimming, there have not been prospective epidemiological studies to actually support this association. Use serology (also consider collecting saliva and possibly fecal

samples) to help identify the etiological agents from sewage that are impacting on recreational water sites (**high priority**).

   a. Conduct similar studies in recreational waters (above refers to studies in sewage) (**medium priority**).

9. Systematically identify and evaluate more reproducible, accurate, and cost effective methods to sample and identify priority pathogens or their index organisms (including the total adenoviruses, [e.g., Groups A-F and adenovirus 40/41], but also JC virus, and Norovirus) in ambient waters (**medium priority**).
10. Determine if there are any appropriate sewage associated bacterial pathogens that can adequately serve as an index of any of the currently known sewage-borne bacterial organisms to use on a more routine basis in recreational water criteria. For example, determine if monitoring recreational waters for *Salmonella* spp. bacteria and phages of *Salmonella* can fulfill the criteria of a pathogen index for sewage-borne bacterial pathogen can be developed (**medium/low priority**).
11. Conduct microbial fate and transport studies to determine relationships between traditional and new fecal indicators, index pathogens, and priority pathogens in treated effluents and in downstream recreational waters to compare and validate their applicability for specific criteria uses (**high/medium priority**).

2.8.2 Longer-term Research Goals

The research below may take longer than 2 to 3 years of research to complete. These are *not* presented in order of priority.

1. Review archived samples to look for trends in evolution of viruses (new or cyclic re-emergence of viruses) and the efficacy of current indicator targets used by molecular methods for health based correlations.
   a. Develop predictive models to understand the conditions that promote the emergence or re-emergence of new pathogens.
2. Continue to conduct additional epidemiological studies on non-point sources of fecal contamination and assess illness relationships to pathogen/indicators.
3. Continue to conduct sewage surveillance for pathogens as a means of public health surveillance and informing pathogen monitoring programs for CWA purposes.
4. Develop robust method for speciation of enterococci with a view to identify fecal-specific (preferably human) from environmental strains; then apply to future MST and epidemiological studies (assuming initial studies suggest that this should be explored further).
5. Conduct studies on beaches to characterize the usefulness of total adenoviruses (Groups A-F), adenovirus 40/41, JC virus, and Norovirus to meet recreational water quality criteria purposes.
6. Conduct health/epidemiological studies to identify a range of pathogens/indicators from various nonhuman sources of fecal contamination.

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EXHIBIT GG

GUAM WATERWORKS AUTHORITY

GWA COMPLIANCE LABORATORY
DEDEDO, GUAM
PHONE: (671) 637-2895/ 632-9697
FAX: (671) 637-2592

SHORELINE MONITORING STATIONS

Site A: Located west of the access road, at the park by the old Diamond Auto.
Site B: Located at the second bridge on the access road to the plant.
Site C: Located at the channel to the boat basin on the Paseo side.
Sited D: Located at the Hagatna River, near Bank of Guam.

ANALYSIS PERFORMED: Fecal Coliform
SAMPLED ON: 02/26/09
TESTED ON: 02/26/09
PERFORMED BY: N. GUTIERREZ AND V. MESA

ENTEROCOCCI

LOCATION	CFU'S/100 mL
SITE A	0
SITE B	0
SITE C	0
SITE D	308

ANALYSIS PERFORMED: E. COLI/ TOTAL COLIFORM
SAMPLED ON: 02/26/09
TESTED ON: 02/26/09
PERFORMED BY: N. GUTIERREZ AND V. MESA

	E. COLI	TC
LOCATION	CFU'/100 mL	CFU'/100 mL
SITE A	491	9208
SITE B	<10	<10
SITE C	<10	<10
SITE D	<10	<10

ANALYSIS PERFORMED: ENTEROCOCCI
SAMPLED ON: 02/26/09
TESTED ON: 02/26/09
PERFORMED BY: N. GUTIERREZ AND V. MESA

ENTEROCOCCI

LOCATION	CFU'/100 mL
SITE A	10
SITE B	<10
SITE C	<10
SITE D	63

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AGANA SHORELINE MONITORING STATIONS

Site A: Located west of the access road, at the park by the old Diamond Auto.
Site B: Located at the second bridge on the access road to the plant.
Site C: Located at the channel to the boat basin on the Paseo side.
Sited D: Located at the Hagatna River, near Bank of Guam.

ANALYSIS PERFORMED: Fecal Coliform
SAMPLED ON: 04/16/09
TESTED ON: 04/16/09
PERFORMED BY: N. GUTIERREZ AND A. DUENAS

Fecal Coliform	
LOCATION	CFU'S/100 mL
SITE A	5
SITE B	0
SITE C	2
SITE D	218

ANALYSIS PERFORMED: E. COLI/ TOTAL COLIFORM
SAMPLED ON: 04/16/09
TESTED ON: 04/16/09
PERFORMED BY: N. GUTIERREZ AND A. DUENAS

	E. COLI	TC
LOCATION	CFU/100 mL	CFU/100 mL
SITE A	468	5794
SITE B	536	4106
SITE C	341	2809
SITE D	>24192	>24192

ANALYSIS PERFORMED: ENTEROCOCCI
SAMPLED ON: 04/16/09
TESTED ON: 04/16/09
PERFORMED BY: N. GUTIERREZ AND A. DUENAS

ENTEROCOCCI	
LOCATION	CFU/100 mL
SITE A	<10
SITE B	<10
SITE C	<10
SITE D	122

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AGANA SHORELINE MONITORING STATIONS

Site A: Located west of the access road, at the park by the old Diamond Auto.
Site B: Located at the second bridge on the access road to the plant.
Site C: Located at the channel to the boat basin on the Paseo side.
Site D: Located at the Hagatna River, near Bank of Guam.

ANALYSIS PERFORMED: Fecal Coliform
SAMPLED ON: 05/14/09
TESTED ON: 05/14/09
PERFORMED BY: N. GUTIERREZ AND V. MESA

Fecal Coliform	
LOCATION	CFU/100 mL
SITE A	4
SITE B	0
SITE C	2
SITE D	44

ANALYSIS PERFORMED: E. COLI/ TOTAL COLIFORM
SAMPLED ON: 05/14/09
TESTED ON: 05/14/09
PERFORMED BY: N. GUTIERREZ AND V. MESA

	E. COLI	TC
LOCATION	CFU/100 mL	CFU/100 mL
SITE A	484	6893
SITE B	548	6015
SITE C	138	2415
SITE D	9208	>24192

ANALYSIS PERFORMED: ENTEROCOCCI
SAMPLED ON: 05/14/09
TESTED ON: 05/14/09
PERFORMED BY: N. GUTIERREZ AND V. MESA

ENTEROCOCCI	
LOCATION	CFU/100 mL
SITE A	<10
SITE B	<10
SITE C	<10
SITE D	341

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AGANA SHORELINE MONITORING STATIONS

Site A: Located west of the access road, at the park by the old Diamond Auto.
Site B: Located at the second bridge on the access road to the plant.
Site C: Located at the channel to the boat basin on the Paseo side.
Site D: Located at the Hagatna River, near Bank of Guam.

ANALYSIS PERFORMED: Fecal Coliform
SAMPLED ON: 06/11/09
TESTED ON: 06/11/09
PERFORMED BY: N. GUTIERREZ AND V. MESA

Fecal Coliform	
LOCATION	CFU/100 mL
SITE A	3
SITE B	0
SITE C	0
SITE D	168

ANALYSIS PERFORMED: E. COLI/ TOTAL COLIFORM
SAMPLED ON: 06/11/09
TESTED ON: 06/11/09
PERFORMED BY: N. GUTIERREZ AND V. MESA

	E. COLI	TC
LOCATION	CFU/100 mL	CFU/100 mL
SITE A	449	6867
SITE B	958	6488
SITE C	381	3968
SITE D	3169	>24192

ANALYSIS PERFORMED: ENTEROCOCCI
SAMPLED ON: 06/11/09
TESTED ON: 06/11/09
PERFORMED BY: N. GUTIERREZ AND V. MESA

ENTEROCOCCI	
LOCATION	CFU/100 mL
SITE A	<10
SITE B	<10
SITE C	<10
SITE D	379