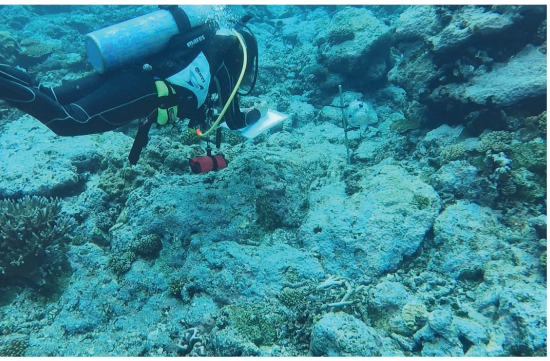
HTTPS://DOI.ORG/10.25923/5VJ3-0225

Assessment of Contamination in Fagatele Bay (National Marine Sanctuary of American Samoa)



May 2022

NOAA TECHNICAL MEMORANDUM NOS NCCOS 303

NOAA NCCOS Stressor Detection and Impacts Division

Citations

Whitall, D., L. Webster, A. Mason, M. Martínez-Colón, M. Sudek, H. Spathias, H. Hattori, L. May, J. Guyon, C. Woodley. 2022. Assessment of Contamination in Fagatele Bay (National Marine Sanctuary of American Samoa). NOAA Technical Memorandum NOS NCCOS 303. Silver Spring. 75 pages.

Acknowledgments

We would like to thank the staff of the National Marine Sanctuary of American Samoa for valuable discussions and logistical support, without which this work would not have been successful. Additionally, we thank Kelley Anderson Tagerino and Francis Leiato of American Samoa Community College, the staff of the American Samoa Environmental Protection Agency, and Noriko Shoji (NOAA-National Marine Fisheries Service, Honolulu) and Paulo Maurin (NOAA-Coral Reef Conservation Program, Honolulu) who provided additional logistical support. We also acknowledge the expert seamanship of Russ Cox (Pago Pago Marine Charters) who piloted the research vessel during field operations. We appreciate the laboratory assistance and expertise of Carl Miller, Zachary Moffitt, Emily Pisarski, Ron Kothera and Murphy McDonald at NCCOS' facility in Charleston, SC. This work was funded by NOAA's Coral Reef Conservation Program, with support from the National Centers for Coastal Ocean Science and the Office of National Marine Sanctuaries. We also thank the following reviewers for their helpful comments that greatly improved the manuscript: Valerie Brown (NOAA-National Marine Sanctuaries), and Tony Pait and Ed Wirth (NOAA-National Centers for Coastal Ocean Science).

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https://doi.org/10.25923/5vj3-0225

Assessment of Contamination in Fagatele Bay (National Marine Sanctuary of American Samoa)

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[5]Coral Reef Conservation Program, Office for Coastal Management, National Ocean Service, National Oceanic and Atmospheric Administration.
[6] Consolidated Safety Services, Inc, contract to NOAA

May 2022

NOAA Technical Memorandum NOS NCCOS 303

United States Department of Commerce	National Oceanic and Atmospheric Administration	National Ocean Service
Wilbur L. Ross, Jr. Secretary	Neil A. Jacobs, PhD Under Secretary	Nicole LeBoeuf Assistant Administrator, Acting

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Abstract

Fagatele Bay is an embayment within the National Marine Sanctuary of American Samoa for which there are minimal data regarding contaminant distribution in this protected area. Resource managers have significant concerns about the potential inputs of contaminants from an unlined, solid waste landfill located approximately 600 m upslope from the Bay. Leachate from the landfill potentially includes both organic (e.g. PCBs, PAHs, personal care products) and inorganic (e.g. heavy metals) pollutants, and could reach the Bay through surface runoff, or, given the permeability of the volcanically derived soils, through groundwater flux. There is also the potential for other land-based sources of pollution (LBSP, such as pesticides) to reach the Bay.

The treatment of solid waste is a serious problem on most islands that can result in toxic substances entering the coastal environment. The potential transport of pollutants from the landfill to the Bay has not been previously quantified. This study addresses this important research question, i.e. what contaminants are present in Fagatele Bay? This assessment is important for two reasons: 1) to determine the extent (magnitude and spatial distribution) of pollution in the Bay; and 2) to serve as baseline for future assessment, and to evaluate the effectiveness of future watershed management activities which might be designed to improve coral reef ecosystem health by reducing LBSP. The approach presented here assessed contamination risk to the Bay using multiple techniques: active in situ water samplers for organic chemistry analysis, metals analysis of sediment samples, bacterial (Colitag) and nutrient analyses of bottom water discrete samples, sea urchin embryo development toxicity assays using SPE-concentrated site water, application of the Salmonella typhimurium reverse mutation assay (Ames test) for mutagenic activity of SPE-concentrated site water, and analysis of foraminifera populations as an indicator of stress. This study found 32 organic pollutants at detectable levels in the Bay. These were all at relatively low concentrations that are unlikely to be of acute toxicological concern. With the exception of nickel, sediment metal concentrations were below previously published Sediment Quality Guidelines, indicating that toxicity to benthic infauna is unlikely. Laboratory toxicity testing of Fagatele Bay samples did not show significant toxicity using the sea urchin embryo development toxicity assay. None of the sample extracts analyzed exhibited mutagenicity via the Ames test (strains TA98 or TA100). Six out of ten water samples tested positive for Escherichia coli, and all samples tested positive for total coliform (Colitag test) demonstrating that mammalian (possibly human) or avian waste is reaching the Bay. Examining the population of benthic foraminifera (FoRAM Index) was not conclusive, perhaps because of the extremely coarse substrate which limited the number and variety of forams collected. Overall, these methods suggest that while some pollutants are reaching the Bay, the water quality of the system is relatively good. Resource managers can use these data as a baseline to ensure that water quality does not degrade over time, and to be aware of specific pollutant groups (e.g. pharmaceuticals) that might be of emerging concern.

Introduction

Contaminant Stressors in Coral Reef Ecosystems

Coral reef ecosystem health can be adversely impacted by a variety of pollution stressors, including nutrients, pathogens, metals, legacy organic pollutants (e.g. PCBs), legacy and current use pesticides, hydrocarbons, flame retardant compounds (PBDEs), personal care products and pharmaceuticals. Numerous NOAA studies have previously quantified the extent and magnitude of pollution in coral reef ecosystems in US waters (e.g. Pait et al. 2007, Mason and Whitall 2019), including in sediments (Hartwell et al. 2017), in the water column (Whitall et al. 2019), in coral tissues (Whitall et al. 2016a) and in motile benthic reef organisms (Whitall et al. 2016b). However, each reef ecosystem has its own unique stressor profile, meaning that individual assessments are required for systems of special interest, such as marine protected areas.

Additionally, merely quantifying the presence of pollutants is not sufficient to understand the biological effects of stressors on the system. This study employs not only multiple methods for detecting pollutants, but also multiple methods of assessing their potential impacts on the reef ecosystem. This multi-pronged approach is described below.

Approach

This study used multiple methodologies to determine the presence and impact of contaminant stressors on the coral reef ecosystem of Fagatele Bay. These methodologies were:

1) Quantification of organic pollutants in the water column using in situ active samplers;
2) Quantification of metals in surface sediments;
3) Sampling and analysis of bottom water (near reef) nutrients;
4) Binary detection (presence/absence) of fecal indicator bacteria;
5) Laboratory determination of potential mutagenic properties of site water (Ames test);
6) Laboratory determination of potential toxicity of site water (sea urchin embryo development assay);
7) Application of the Foram Index as a water quality indicator.

By considering the results from all of these methods together, in a preponderance of evidence approach, the conclusions that can be made from these data are much stronger than by considering any one method alone.

In Situ Active Water Sampling for Organic Pollutants

Quantifying the concentrations of over 400 chemical contaminants in the environment allows us to describe the nature of the pol¬lution present, make hypotheses about their sources and fate, and begin to document potential hazards in this area associated with the reported chemical concentrations. Each class of contaminant detected during this project is discussed below in the Results and Discussion section. A more detailed discussion of the full contaminant list (including contaminants not detected in this study) is available in Mason and Whitall (2019).

Because the concentrations of water column constituents change over short time periods (e.g. due to tides, currents, land-based runoff), natural integrators of ambient pollution, such as sediments or biota, are often used rather than discretely collected site water. Chemical analysis of biological tissue samples, such as fish or macroinvertebrates, can be informative, but studies can be limited by the abundance or harvestability of the target organism, and data interpretation can be confounded by the movement of animals within the system, and the ability of organisms to uptake and depurate contaminants from their tissues. Although sediments typically serve as a reservoir for chemical contaminants that can bioaccumulate or biomagnify in aquatic organisms, very sandy (and coarser) sediments (such as those found in coral reef ecosystems) are poor integrators of organic contaminants over time. Additionally, coral reef ecosystems with high levels of hard bottom cover (reefs or pavement) may not have much sediment available for sampling. Furthermore, some

water-soluble compounds (e.g. current use pesticides, personal care products) do not accumulate in sediments or tissues due to their hydrophilic nature.

To attempt to address some of the shortcomings of discrete water sampling and the use of natural integrators, researchers have developed passive sampling devices such as Polar Organic Chemical Integrative Sampler (POCIS; Alvarez et al., 2004), PolyEthylene Devices (PEDs, Lohmann 2012) and silicon bands (Swanson et al. 2018). These in situ passive devices all work in a similar manner; ambient organic pollutants adhere to the sampling matrix over time. After a field deployment period (usually on the order of 30 days), the device can be retrieved from the field and the pollutants extracted and quantified in the laboratory. However, these passive device methods only yield a mass of analyte that has adhered to the sampling matrix, not actual concentrations. Ambient concentrations can be estimated using laboratory-derived rate constants if there is a targeted understanding of the rates by which chemicals bind to the sampling device (i.e. the linear rate of chemical / device binding or the steady-state relationship of the chemical and device); any uncertainty in the expression of this relationship adds to the uncertainty of the measurement. It should be noted that these rate constants may not be available for every contaminant, especially contaminants of emerging concern, which would require additional laboratory work on the part of the investigators to develop these constants.

An alternative to both traditional field methods (water, sediment, tissue sampling) and passive samplers are in situ active samplers, such as the Continuous Low-level Aquatic Monitoring Devices (CLAMs; Aqualytical Inc, Louisville, KY). This unit uses a similar matrix (semi-permeable membrane; HLB or C18 filter) to the POCIS passive samplers, but includes a pump which actively pumps a known volume of water across the filter. Because the volume of water sampled is known, the concentration can be directly calculated rather than modeled using rate constants. It should be noted that not all potential contaminants will sorb to either filter type, but the combination of the two filter types yields an extensive list of targeted analytes. Another advantage of CLAMs over discrete samples is that CLAMs can sample large volumes (>75L) of water in one 24-hour deployment. This allows for much lower levels of detection than would be possible from a traditional 1L discrete sample and does not require month long deployment times, which can put equipment at risk due to loss or vandalism, or may not be conducive to field logistics at a remote field site. Additional details on the CLAM devices are presented in the Methods section (below).

Sediment Sampling for Trace and Major Elements Analysis

As discussed above, sediments tend to accumulate contaminants, including metals. Because the CLAM filters only accumulate organic pollutants, sediments can be used in parallel with CLAM samples in order to capture metal concentrations in the system of interest.

Nutrients and Fecal Indicator Bacteria in Bottom Water

Discrete samples were collected for nutrient analysis at each site. Samples were analyzed for nitrate, nitrite, ammonium, urea, organic nitrogen, orthophosphorus, total phosphorus and silica. Because singular discrete samples do not capture the variability of a system (see discussion above), these data must be viewed as a “snapshot” of nutrient conditions in the Bay. A more rigorous (e.g. at least monthly) sampling program would need to be conducted in order to better assess the nutrient status of the Bay. See Whitall et al. (2019) for an example of a more rigorous nutrient study on another bay (Vatia) in American Samoa. In addition to nutrient analysis, indicators of bacterial pollution were tested at each CLAM site and the freshwater waterfall that flows downhill into the Bay. Total coliform and E. coli presence/absence data provided additional water quality information.

Assessment of Mutagenicity

Traditionally, the Ames test (a.k.a. the bacterial reverse mutation assay with and without activation) has been used as an indicator of mutagenic effects of newly developed chemicals (Maron and Ames 1983, Mortelmans and Zeiger 2000). The test has also been used to detect mutagenic compounds in drinking water (Vughs, 2018, Guan, et al, 2017, Sujbert, 2006), wastewater (Abbas et al 2019, Tabet et al 2015), river and surface water (Roubicek, 2020, Xiao, 2017, Sueiro, 2011, Wu, 2005, Vargas et al, 1993), sediment pore water (Parella, 2013), swimming pools (Manasfi, et al, 2016), textile effluent (Vacchi, 2017), cigarette smoke (Thorne, 2015), biochars and ash from an incinerator (Piterina, 2017, Chen, 2015), and the mutagenicity of the UV filter (sunscreen) benzophenone and related compounds (Wang et al, 2018). The Ames test employs genetically modified strains of Salmonella typhimurium with mutations in the histidine operon, that disable histidine production. Each strain has a specific mutation (i.e., deletion and frame shift or a base substitution) in susceptible regions of the his gene that are sensitive to reversion by certain classes of chemicals. Exposure of test

strains to a mutagen, engages the error-prone DNA repair system, allowing mutations that revert the test strain's mutation to wild-type and allow growth on media without histidine (i.e., allows histidine production). A small number of bacteria will revert naturally during their growth phase. Strains TA98 and TA100 are often used for screening. TA98 was engineered with a deletion resulting in a frameshift mutation (Mortelmans and Zeiger, 2000), while TA100 has a base pair substitution mutation (Mortelmans and Zeiger, 2000). These two test strains were used to screen for mutagenic activity water concentrated by SPE columns from the Fagatele Bay as an indicator of water quality on coral reefs.

Water Toxicity Using Sea Urchin Embryo Exposures

Echinoid species are commonly found in nearshore marine habitats across the globe. The ubiquitous nature of sea urchins and the well-defined developmental scheme of urchin embryos make them useful test organisms for estimating toxicity. Since early life stages can be particularly sensitive to environmental aberrations, sea urchin embryos have been used to assess toxicity to various test materials including sediment interstitial waters (porewater) (Carr and Chapman 1992; Carr et al. 1996), effluents and receiving waters (Weber et al. 1988), and to evaluate the potential toxicity of various chemical contaminants (Hamdoun et al. 2002; Manzo et al. 2006; Rock et al. 2011; Rouchon and Phillips 2017). A standard protocol (ASTM, 1998) with relevant tropical urchin species (Lytechinus variegatus, Tripneustes gratilla, Echinometra sp.) was used to evaluate potential toxicity associated with nutrient pollution and emerging contaminants of concern. Sampled sediments did not yield sufficient porewater volumes to perform the test; therefore, ambient seawater was collected and filtered over solid phase extraction (SPE) columns. Column eluates (in dimethylsulfoxide) were reconstituted in artificial seawater for the assay to gauge toxicity at each site. While this method has not been previously described for assessing the toxicity of environmental samples using the sea urchin embryo development test, SPE columns are routinely used to bind select contaminants for seawater chemistry analysis.

Application of the Foram Index as a Water Quality Indicator

Ecological indicators are used to assess environmental conditions as well as trends over time (Dale and Beyeler, 2001). As calcareous marine protozoans found in virtually all marine ecosystems, benthic foraminifera have been used as ecological indicators since 1950 (Sen Gupta, 2013). These cosmopolitan shelled-protists are very sensitive to environmental changes (e.g., turbidity, pH, organic matter, heavy metals, etc.); hence they integrate the environment's cumulative physiochemical conditions (Castelo et al., 2021; Martínez-Colón et al., 2018), and any changes in distribution, assemblage, and species dominance are a direct result of environmental changes. Their rapid ecological response can be measured by assessing changes in community structure, density, faunal turnovers, and dominance of key stress-tolerant taxa. In addition, the spatial and temporal variability of benthic foraminifera are a direct response to external (abiotic) and/or internal (biotic) stressors leading to changes in species composition (Schafer, 2000).

Numerous ecological indices have been developed using benthic foraminifera. These range from deep ocean settings to assess environmental health conditions (e.g., Foram-AMBI; Alve et al., 2016) to determining redox conditions in estuarine settings (Ammonia-Elphidium index; Sen Gupta and Platon, 2006). In coral reefs, the foraminiferal community structure is controlled by the same abiotic factors (e.g., temperature, light penetration, nutrient flux) as their coral counterparts. An added advantage of reef-dwelling foraminifers is that they are more sensitive and react faster to an environmental stressor than corals (Oliver et al., 2014). The FoRAM Index (Foraminifers in Reef Assessment and Monitoring; FI) was developed as a water quality indicator in reefs systems in order to determine the suitability of the environment for reef structure development, including the potential for reef recovery (e.g., coral recruitment or nursery transplants) following a stress event (Prazeres et al., 2020; Hallock, 2012; Hallock, et al., 2003). The FI is a very simple single-metric index based on the assemblage of reef–associated benthic foraminifers. For example: symbiont-bearing reef-dwelling foraminifers thrive in healthy reefs influenced by clear oceanic-waters with scarce food, and dominate the assemblage (e.g., Hallock et al., 2003). On the other hand, smaller heterotrophic taxa and stress-tolerant (e.g., opportunistic) taxa thrive in conditions where light penetration is not a limiting factor but variability in food sources (labile organic matter) (Uthicke and Nobes, 2008) and changes in salinity and oxygenation are limiting (e.g., Prazeres et al., 2020). In addition, FI has been demonstrated to reflect substrate type, distance from shore, algae and coral cover changes (e.g., Barbosa et al., 2009; Emrich et al., 2017).

Study Site

Fagatele Bay is located on the south shore of the island of Tutuila, the largest and most populous island of the U.S. territory of American Samoa (). Fagatele Bay National Marine Sanctuary was established in 1986 to protect the unique coral reef ecosystem located in this remote bay. The sanctuary was expanded in 2012 and renamed the National

Figure 1: Location of American Samoa and location of Fagatele Bay in American Samoa.

Marine Sanctuary of American Samoa. It is one of 15 sites in NOAA's National Marine Sanctuary System. American Samoa's reefs are considered to be among the most pristine in the United States (Birkeland et al. 2008); these reefs host approximately 950 species of fish, 240 species of algae, 330 species of coral and many other species of invertebrates (Birkeland et al. 2008). Fagatele Bay's reefs are considered to be in very good condition due to its relatively remote location (NMSP 2007). The Bay is roughly horseshoe shaped and is approximately 0.6 km wide at its widest point. The Bay has a high degree of hard bottom habitat (live coral, pavement) with some patches of calcium carbonate, sandy sediment (diver observations, 2019) and its opening to the ocean faces the south-southwest. There is very little development in the watershed, with a handful of residences and small agricultural plots scattered across the landscape. A likely potential source of pollution is the Futiga landfill, an unlined solid waste landfill that serves the entire island. The landfill has been used as a municipal waste disposal site since the 1960s. It was recompacted in 2018 to extend its lifespan, but is nearing its capacity. The lack of a liner and leachate collection system has caused concern about potential contamination to adjacent waters. Polidoro et al. (2017) quantified concentrations of heavy metals, pesticides and PAHs in seven coastal streams near the Futiga landfill. All sampled stream sediments contained high concentrations of lead, and some contained high mercury concentrations. Water samples from several coastal streams showed relatively high concentrations of organophosphate pesticides, above chronic toxicity values for fish and other aquatic organisms. Although it was banned in 2006, the pesticide parathion was also detected in several stream sites. This previous work suggests that there is some source of toxic materials within the Fagatele watershed. Additionally, given the porous nature of the soils (igneous source material; USDA 1984), groundwater transport of pollutants to the coastal zone is also a concern.

Methods

To assess the potential impact of the adjacent landfill and agricultural activities on Fagatele Bay, CLAM samplers were deployed. CLAM samplers (Figure 2) pump a known volume of water across specialized membranes (HLB and C18) which capture the contaminants for subsequent analysis in the laboratory. The sites were selected in a targeted manner, roughly following the shoreline of the Bay (Figure 3; Table 1) to maximize the likelihood of capturing the potential groundwater signal coming from land. The CLAMs were deployed for 24 hours at a time in order to integrate the temporal variability in the system and not "miss" key events (e.g. tides, currents, precipitation). In April 2019, CLAM units were deployed on the bottom (attached with zip ties to rebar that had been driven into the pavement) at eight reef

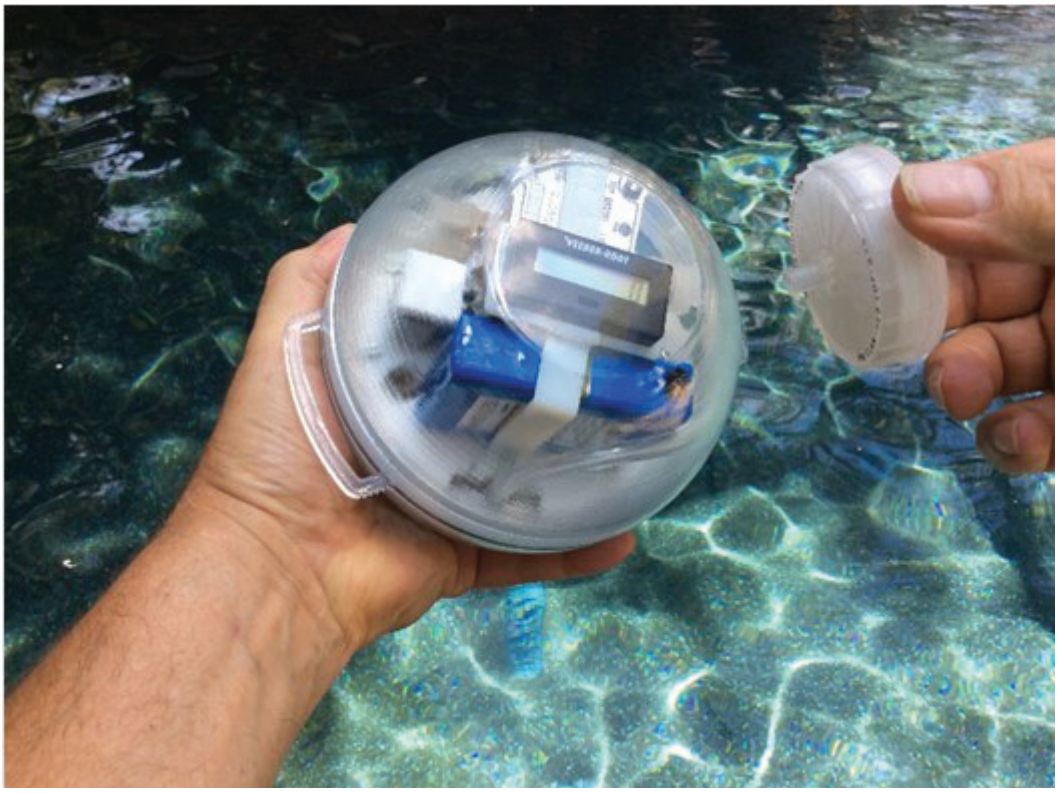

Figure 2: Photo of CLAM sampling device.

Table 1: List of Sampling Sites in Fagatele Bay. See also Figure 3.

Site	Latitude	Longitude	Depth (m)	Analysis
1	-14.3647	-170.7610	4	CLAM, Nutrients, Toxicity, Ames, Colitag, Foram, TOC, grain size
2	-14.3644	-170.7609	7	CLAM, Nutrients, Toxicity, Ames, Colitag, Foram, TOC, grain size
3	-14.364	-170.7612	7	CLAM, Nutrients, Toxicity, Ames, Colitag
4	-14.3637	-170.7615	6	CLAM, Nutrients, Toxicity, Ames, Colitag, Foram, TOC, grain size
5	-14.3634	-170.7618	5	CLAM, Nutrients, Toxicity, Ames, Colitag, Foram, TOC, grain size
6	-14.3662	-170.7621	7	CLAM, Nutrients, Toxicity, Ames, Colitag, Foram, Sediment Chem
7	-14.3659	-170.7618	5	CLAM, Nutrients, Toxicity, Ames, Colitag, Foram, TOC, grain size
8	-14.3629	-170.7625	6	CLAM, Nutrients, Toxicity, Ames, Colitag, Foram, TOC, grain size
9	-14.3646	-170.7607	4	Nutrients, Toxicity, Ames
SE	-14.3665	-170.7623	7	Sediment chemistry, TOC, grain size
SF	-14.366	-170.7619	7	Sediment chemistry, TOC, grain size
SG	-14.3644	-170.7610	10	Sediment chemistry, TOC, grain size
SH	-14.3643	-170.7611	9	Sediment chemistry, TOC, grain size
A	-14.3654	-170.7611	5	Foram, TOC, grain size
C	-14.3641	-170.7610	8	Foram, TOC, grain size

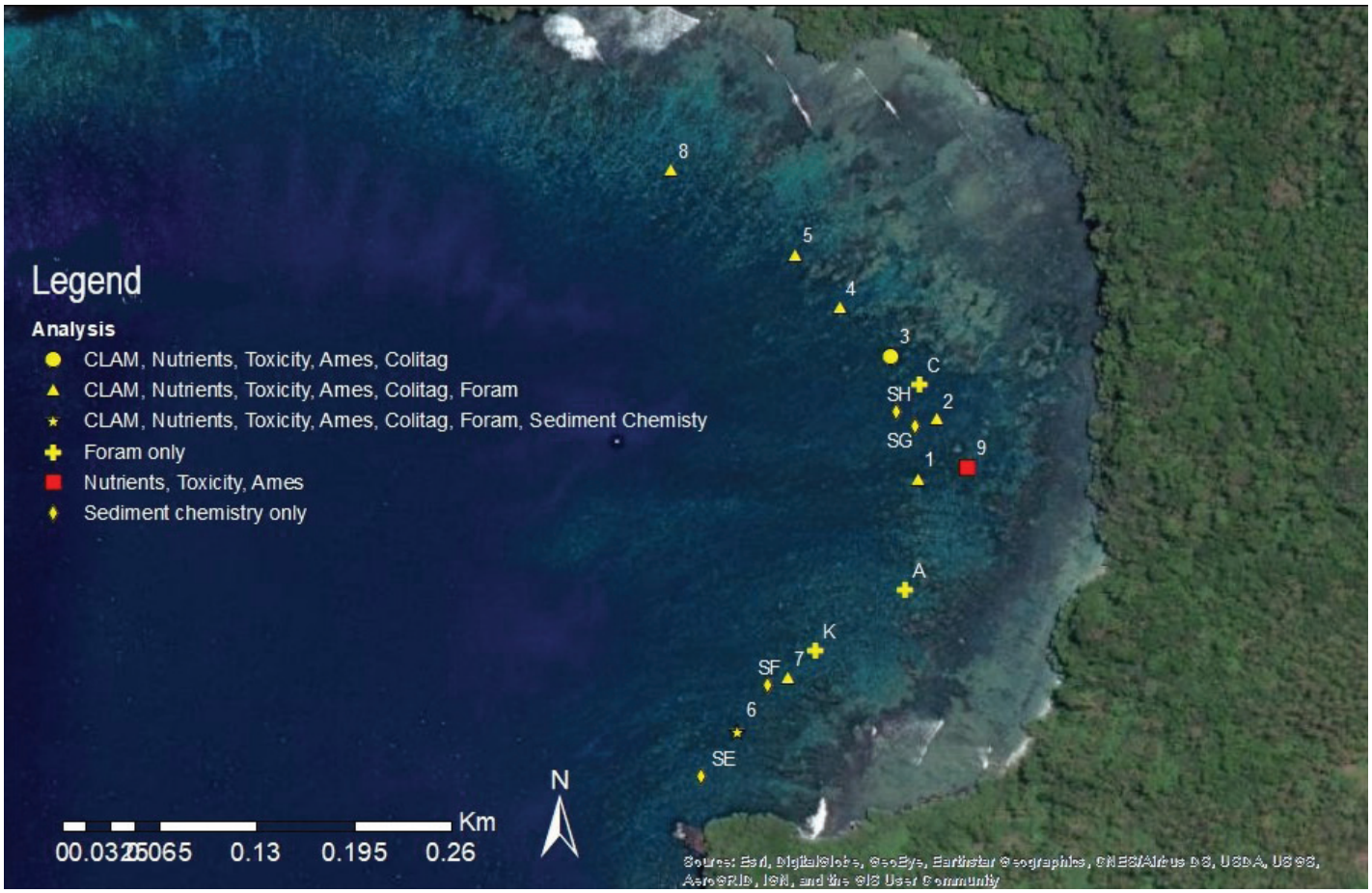

Figure 3: Map of sampling sites and the analyses associated with each.

sites within the Bay (Figure 4). Each filter type (HLB vs C18) was deployed twice (two 24 hours periods) at each site and the two filters of each type were composited for analysis so that there was one concentration value generated for each analyte per site. Each value represented an integrated concentration reflecting between 68 and 245 liters of water over that 48 hour period. CLAM filters were analyzed for over 400 organic contaminants (Table 2a,b). Additionally, at each CLAMs site where there was sediment (sand), a small amount of material was collected into certified clean I-Chem glass jars for metals analysis bcause C18 and HLB filters do not capture metals. If sand was not available at exactly the same site as the CLAM, nearby sediment was collected when possible and is reflected with a different site name. Chemistry analyses were conducted under contract at TDI Brooks (College Station, TX) and SGS AXYS (Vancouver, BC) laboratories. TDI Brooks methods are described in detail in Kimbrough et al. (2006 and 2007). Laboratory analysis methods specifically for AXYS related analytical results (current use pesticides and human use pharmaceuticals) are proprietary and confidential. The method names used for this study were MLA-035 REV.07.04 and MLA-070 REV.07.04. Contact information for further references is: SGS-AXYS Analyti¬cal Services Ltd, 2045 Mills Road W., Sidney, BC, Canada, V8L 5X2. Tel. (250) 655-5800, fax (250) 655-5811. Field staff wore nitrile gloves during sampling to prevent cross contamination of samples.

Discrete samples of bottom water for nutrient analysis were collected by SCUBA divers at each of the CLAM sites (Figure 3; Table 1). High density polyethylene (HDPE) bottles, pre-cleaned and rinsed three times with site water, were used for sample collections. Samples were stored on ice, in the dark while in the field, frozen at -20oC upon returning to the lab at the end of each field day, and not thawed until immediately prior to analysis. Water samples were not filtered so that total nutrient levels could be analyzed, rather than only dissolved levels. Samples were analyzed for: nitrate, nitrite, ammonium, urea, total nitrogen, orthophosphate, total phosphorus and silica via standard methods (Armstrong et al 1967, Bernhardt and Wilhelms 1967, Harwood and Kuhn 1970, Hansen and Koroleff 1999) at a NOAA contract laboratory (Geochemical and Environmental Research Group, Texas A&M University, College Station, TX).

Water samples (100 mL each) were collected from each CLAM site, from the primary waterfall, Site W, and a thin stream of water naturally diverted from the primary waterfall across a rock wall (Figure 5), Site T, as well as bottled spring water, were collected in 100 mL sterile bottles and analyzed for the presence of total coliforms and E. coli in water samples

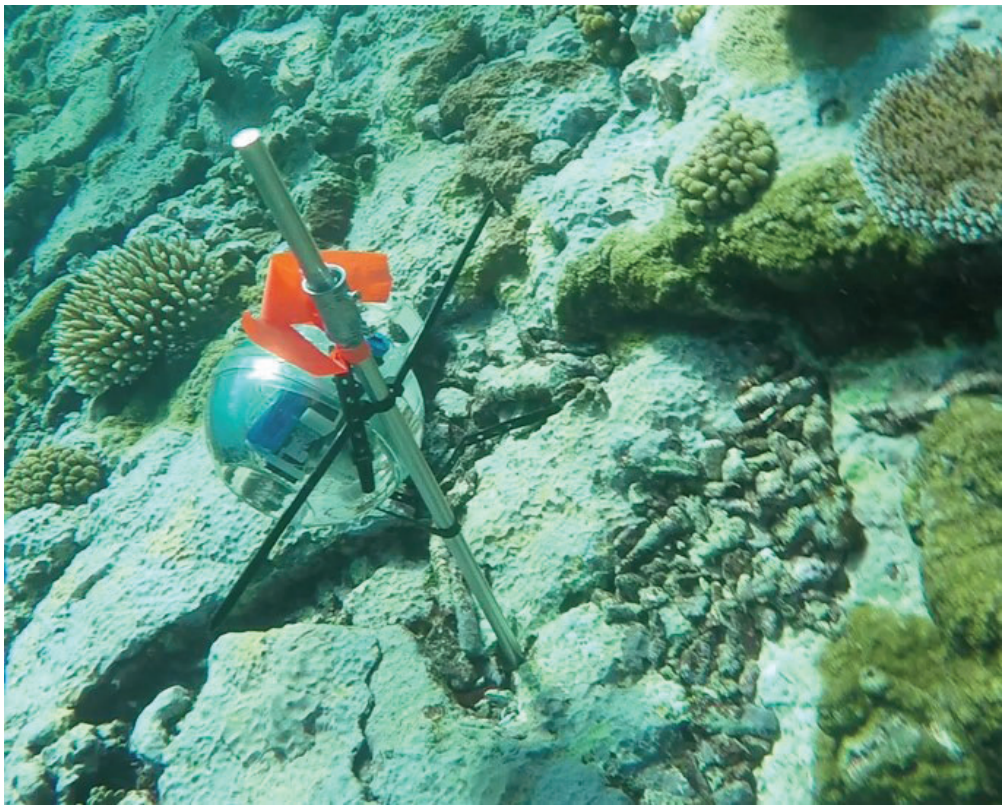

Figure 4: Photo of deployed CLAM device in Fagatele Bay.

Figure 5: Waterfall draining into Fagatele Bay

Table 2a: Analytes quantified on CLAM filters. Note: not all of these compounds were detected. Please see Table 3 and 3b.

Aldrin	HCH, gamma	PCB 6	PCB 76/70	PCB 134/133
alpha-Endosulphan	Heptachlor Epoxide	PCB 8/5	PCB 66/80	PCB 165/131
Atrazine	Hexazinone	PCB 14	PCB 55	PCB 142/146/161
beta-Endosulphan	Linuron	PCB 11	PCB 56	PCB 153/168
Chlordane, oxy-	Malathion	PCB 12	PCB 60	PCB 132
Desethylatrazine	Methoxychlor	PCB 13	PCB 79	PCB 141
Endrin Ketone	Metolachlor	PCB 15	PCB 78	PCB 137
HCH, beta	Metribuzin	PCB 19	PCB 81	PCB 130
Heptachlor	Nonachlor, cis-	PCB 30	PCB 77	PCB 138/164/163
Hexachlorobenzene	Nonachlor, trans-	PCB 18	PCB 104	PCB 160/158
Mirex	Octachlorostyrene	PCB 17	PCB 96/103	PCB 129
Simazine	Parathion-Ethyl	PCB 27	PCB 100	PCB 166
2,4'-DDD	Parathion-Methyl	PCB 24	PCB 94	PCB 159
2,4'-DDE	Pendimethalin	PCB 16/32	PCB 102/98	PCB 162
2,4'-DDT	Permethrin	PCB 34	PCB 121/93/95	PCB 128/167
4,4'-DDD	Perthane	PCB 23	PCB 88	PCB 156
4,4'-DDE	Phorate	PCB 29	PCB 91	PCB 157
4,4'-DDT	Phosmet	PCB 26	PCB 92	PCB 169
Alachlor	Pirimiphos-Methyl	PCB 25	PCB 101/84/90	PCB 188
Ametryn	Quintozene	PCB 28/31	PCB 89/113	PCB 184
Azinphos-Methyl	Tebuconazol	PCB 21/20/33	PCB 99	PCB 179
Butralin	Tecnazene	PCB 22	PCB 119	PCB 176
Butylate	Terbufos	PCB 36	PCB 112	PCB 186/178
Captan	Triallate	PCB 39	PCB 120/83	PCB 175
Chlordane, alpha (c)	Trifluralin	PCB 38	PCB 97/125/86	PCB 187/182
Chlordane, gamma (t)	Endrin Aldehyde	PCB 35	PCB 116/117	PCB 183
Chlorothalonil	Heptachlor-Epoxide	PCB 37	PCB 111/115/87	PCB 185
Chlorpyriphos	Oxychlordane	PCB 54	PCB 109	PCB 174
Chlorpyriphos-Methyl	Alpha-Chlordane	PCB 50	PCB 85	PCB 181
Chlorpyriphos-Oxon	Gamma-Chlordane	PCB 53	PCB 110	PCB 177
Cyanazine	Trans-Nonachlor	PCB 51	PCB 82	PCB 171
Cypermethrin	Cis-Nonachlor	PCB 45	PCB 124	PCB 173
Dacthal	Alpha-HCH	PCB 46/69/73	PCB 106/107	PCB 192/172
Diazinon	Beta-HCH	PCB 52	PCB 123	PCB 180/193
Diazinon-Oxon	Delta-HCH	PCB 43	PCB 118/108	PCB 191
Dieldrin	Gamma-HCH	PCB 49	PCB 114/122	PCB 170/190
Dimethenamid	DDMU	PCB 48/75/47	PCB 105/127	PCB 189
Dimethoate	1,2,3,4-Tetrachlorobenzene	PCB 65	PCB 126	PCB 202
Disulfoton	1,2,4,5-Tetrachlorobenzene	PCB 62	PCB 155	PCB 201
Disulfoton Sulfone	Pentachloroanisole	PCB 44	PCB 150	PCB 204
Endosulphan Sulphate	Pentachlorobenzene	PCB 59	PCB 152	PCB 197
Endrin	Endosulfan II	PCB 42	PCB 148/145	PCB 200

Table 2a continued.

Ethalfluralin	Endosulfan I	PCB 72	PCB 136/154	PCB 198
Ethion	Endosulfan Sulfate	PCB 71	PCB 151	PCB 199
Fenitrothion	Chlorpyrifos	PCB 68/41/64	PCB 135	PCB 203/196
Flufenacet	PCB 1	PCB 40/57	PCB 144	PCB 195
Flutriafol	PCB 2	PCB 67	PCB 147	PCB 194
Fonofos	PCB 3	PCB 58	PCB 149/139	PCB 205
HCH, alpha	PCB 4/10	PCB 63	PCB 140	PCB 208
HCH, delta	PCB 7/9	PCB 61/74	PCB 143	PCB 207

Table 2b: Analytes quantified on CLAM filters. Note: not all of these compounds were detected. Please see Table 3a and 3b.

PCB 206	C1-Chrysenes	Norfloxacin	Trenbolone acetate
PCB 209	C2-Chrysenes	Norgestimate	Valsartan
cis/trans Decalin	C3-Chrysenes	Ofloxacin	Verapamil
C1-Decalins	C4-Chrysenes	Ormetoprim	Cocaine
C2-Decalins	Benzo(b)fluoranthene	Oxacillin	DEET
C3-Decalins	Benzo(k,j) fluoranthene	Oxolinic Acid	Prednisolone
C4-Decalins	Benzo(a)fluoranthene	Penicillin G	Diatrizoic acid
Naphthalene	Benzo(e)pyrene	Penicillin V	Iopamidol
C1-Naphthalenes	Benzo(a)pyrene	Roxithromycin	Citalopram
C2-Naphthalenes	Perylene	Sarafloxacin	Tamoxifen
C3-Naphthalenes	Indeno(1,2,3-c,d) pyrene	Sulfachloropyridazine	Cyclophosphamide
C4-Naphthalenes	Dibenzo(a,h) anthracene	Sulfadiazine	Venlafaxine
Benzothiophene	C1-Dibenzo(a,h) anthracenes	Sulfadimethoxine	Amsacrine
C1-Benzothiophenes	C2-Dibenzo(a,h) anthracenes	Sulfamerazine	Azathioprine
C2-Benzothiophenes	C3-Dibenzo(a,h) anthracenes	Sulfamethazine	Busulfan
C3-Benzothiophenes	Benzo(g,h,i)perylene	Sulfamethizole	Clotrimazole
C4-Benzothiophenes	Bisphenol A	Sulfamethoxazole	Colchicine
Biphenyl	Furosemide	Sulfanilamide	Daunorubicin
Acenaphthylene	Gemfibrozil	Sulfathiazole	Doxorubicin
Acenaphthene	Glipizide	Thiabendazole	Drospirenone
Dibenzofuran	Glyburide	Trimethoprim	Etoposide
Fluorene	Hydrochlorothiazide	Tylosin	Medroxyprogesterone Acetate
C1-Fluorenes	2-Hydroxy-ibuprofen	Virginiamycin M1	Metronidazole
C2-Fluorenes	Ibuprofen	1,7-Dimethylxanthine	Moxifloxacin
C3-Fluorenes	Naproxen	Alprazolam	Oxazepam

Table 2b continued.			
Carbazole	Triclocarban	Amitriptyline	Rosuvastatin
Anthracene	Triclosan	Amlodipine	Teniposide
Phenanthrene	Warfarin	Benzoylecgonine	Zidovudine
C1-Phenanthrenes/ Anthracenes	Acetaminophen	Benztropine	Melphalan
C2-Phenanthrenes/ Anthracenes	Azithromycin	Betamethasone	Albuterol
C3-Phenanthrenes/ Anthracenes	Caffeine	Desmethyldiltiazem	Atenolol
C4-Phenanthrenes/ Anthracenes	Carbadox	Diazepam	Atorvastatin
Dibenzothiophene	Carbamazepine	Fluocinonide	Cimetidine
C1-Dibenzothiophenes	Cefotaxime	Fluticasone propionate	Clonidine
C2-Dibenzothiophenes	Ciprofloxacin	Hydrocortisone	Codeine
C3-Dibenzothiophenes	Clarithromycin	10-hydroxy-amitriptyline	Enalapril
C4-Dibenzothiophenes	Clinafloxacin	Meprobamate	Hydrocodone
Fluoranthene	Cloxacillin	Methylprednisolone	Metformin
Pyrene	Dehydronifedipine	Metoprolol	Oxycodone
C1-Fluoranthenes/Pyrenes	Diphenhydramine	Norfluoxetine	Ranitidine
C2-Fluoranthenes/Pyrenes	Diltiazem	Norverapamil	Triamterene
C3-Fluoranthenes/Pyrenes	Digoxin	Paroxetine	Amphetamine
C4-Fluoranthenes/Pyrenes	Digoxigenin	Prednisone	Cotinine
Naphthobenzothiophene	Enrofloxacin	Promethazine	
C1-Naphthobenzothiophenes	Erythromycin-H2O	Propoxyphene	
C2-Naphthobenzothiophenes	Flumequine	Propranolol	
C3-Naphthobenzothiophenes	Fluoxetine	Sertraline	
C4-Naphthobenzothiophenes	Lincomycin	Simvastatin	
Benz(a)anthracene	Lomefloxacin	Theophylline	
Chrysene/Triphenylene	Miconazole	Trenbolone	

using a Colitag™ test kit (CPI International, Santa Rosa, CA) according to the manufacturer's protocol. Prior to testing the seawater samples, each was diluted 1:10 in the sterile test bottles supplied with the kit with 0.22 µm filter-sterilized (Corning #430513, Corning, NY) spring water, as recommended by the manufacturer.

Filtered sterilized bottled spring water was used as the negative control. Inoculated bottles were incubated overnight (16-20 h) at the outdoor ambient temperature (27°C) and monitored for changes in color. A change in the media from nearly colorless to yellow indicated the presence of total coliforms (Figure 6). Positive samples were placed under UV light (365 nm wavelength) to determine the presence or absence of E coli. Samples that fluoresced blue were considered positive for E. coli.

Replicate seawater samples were collected in pre-cleaned 1 L amber glass bottles (Environmental Express #APC1430, Charleston, SC) for toxicity bioassays (Ames test and sea urchin embryo development). Collection sites were co-located with the eight CLAMs sites in Fagatele Bay (Figure 3; Table 1). A ninth sample (2400 mL) was taken from a diver-observed temperature anomaly located between Sites 1 and 2. The seawater samples were stored on ice until processed after

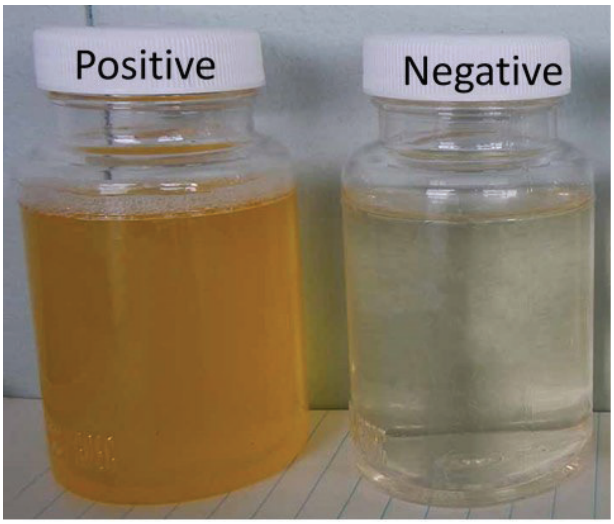

Figure 6: Photo of Colitag test results.

Figure 7: Photo of SPE vacuum manifold and samples.

returning to shore. A total of six bottles of seawater (8400 ml) were collected for each site over the five-day sampling period.

The SPE columns (Oasis HLB columns/cartridges; Waters, Milford, MA) were conditioned at the NCCOS Charleston Laboratory with three column volumes (i.e. 3 x 6 mL) of methanol and rinsed with one volume of ultra-pure water (Bratkovics and Sapozhinikova 2011, USEPA 2007). The column was sealed with Parafilm®, wrapped in acetone rinsed-aluminum foil, then stored refrigerated (4°C) in a zipper lock bag until use. Once on shore, the seawater samples were gradually warmed to room temperature, then filtered through an HLB column (Figure 7) with vacuum (Bratkovics and Sapozhinikova 2011, USEPA 2007).

Two columns were used for each sample site, one column designated for sea urchin embryo development assays (-SUE) and one for Ames test (-mut). Each column was used to concentrate pooled seawater samples from multiple collections from each site. A total of 17 columns were used (8 CLAM sites x 2 columns + 1 column for site 9). The column bed was left damp with seawater after each use. Each column was sealed with Parafilm©, wrapped in clean (acetone-rinsed) aluminum foil, and stored refrigerated in a zipper lock bag until it was used for the next water sample from that site. Each column was warmed to room temperature before being used for subsequent sample collections. After all water samples were filtered, columns were sealed with Parafilm© and foil, and frozen (~ -5°C). The columns remained frozen until extracted at the Charleston Laboratory. One liter of artificial seawater (ASW, Tropic Marin Sea Salts, Wartenburg, Germany, 36 psu) was passed through a conditioned HLB column to be used as a negative control.

The HLB columns were processed at the NCCOS Charleston Laboratory. The columns were warmed to room temperature. Light vacuum was applied to the SPE columns fitted to a column manifold using an oil-less vacuum pump allowing the SPE columns to dry (~ 5 minutes). Solvent (1:1, acetone: methanol) was applied to the columns and elution was initiated with application of a light vacuum. The HLB columns were extracted with three column volumes (i.e. 3 x 6 mL) of solvent. The extracts were each collected in a clean glass tube for each column and then transferred to a round bottom flask. The eluate from the two columns for each site were pooled into one flask to maximize the amount of analyte. The flasks were placed in a TurboVap II (Biotage, Charlotte, NC) to remove the solvents from the extracts. Each sample pellet was suspended with 8.4 mL of dimethyl sulfoxide (DMSO), except for Site #9 which was suspended in 2.8 mL of DMSO (1000X concentration of the sample volume). The reconstituted eluate was stored at 4°C until assayed with the Ames test, then stored at -20°C for the sea urchin embryo development assay.

Each reconstituted sample underwent the Ames test at a single dose representing 100 ml of original seawater sample per agar plate with S. typhimurium TA98 and TA100 (Mortelmans and Zieger, 2000). The assays were conducted with and without metabolic activation (+ and- S9 mix, 10%) to detect direct mutagenic (- S9) and pre-mutagenic (+ S9) compounds requiring metabolic activation. Assays were performed according to the supplier's protocol (Molecular Toxicology Inc, Boone, NC, Appendices IV and V). Briefly, a mixture of 100 µl reconstituted eluate, 2 mL top agar supplemented with bio/his, 500 µL of S9 mix (if used) and 100 µL of overnight bacteria culture (A660 = 1.2 – 1.4) were poured onto the surface of a minimal glucose agar plates. Three plates per sample were tested resulting in a total of 300 mL of seawater

sample evaluated. Positive controls with known mutagenic chemicals and negative controls with the sample solvent (DMSO) were included in the tests. The plates were incubated for 48 h prior to enumeration.

Each plate was photographed using the G:box Imaging System (Syngene, Frederick, MD), illuminated with transmitted light through a sheet of blue acrylic as a filter and saved as a tiff image. Colony enumeration was automated using a Python script (Appendix). The Ames test was considered positive if any of the treatments produced more than twice the number of colonies on the negative control plate.

For the sea urchin embryo development assay, frozen column eluates (in DMSO) from Fagatele Bay and the negative (solvent) control samples (in DMSO) were thawed at room temperature, mixed well and diluted 1:1000 in ASW (50 mL volume) for the assay. An aliquot (5 mL) of each diluted eluate was transferred to a 50 mL sterile polypropylene tube, and salinity and pH were measured to ensure general water quality for the bioassay. Total ammonia nitrogen (TAN) was determined from a 500 µL subsample using a colorimetric microplate assay based on a commercial kit (salicylate method; method detect limit of 0.006 mg/L). Ammonia standards for the assay were generated using 100 mg/L ammonia standard (Hach, Catalog #2406549) in a two-fold dilution series (0.13-8.0 mg/L) in 36 psu artificial ASW. Unionized ammonia (UAN) values were calculated using a standard method (Bower and Bidwell 1978). Measuring these water quality parameters ensures that there are not confounding variables in the toxicity assay. Following water quality analysis, reconstituted samples (5 mL, 4 replicates) were dispensed into pre-cleaned, rinsed (5 mL ASW, 36 psu), 20-mL glass vials (Environmental Express, Charleston, SC) and placed in an environmentally-controlled room (26.0 +/-0.5 °C) to warm.

Toxicity of the reconstituted samples was determined according to a standard method (ASTM, 1998) using a tropical sea urchin species, and as we have reported previously using sediment interstitial water (Balthis et al. 2018; May and Woodley 2016). Gravid sea urchins (Lytechinus variegatus) were acquired from the Florida Keys (Reeftopia, Key West, FL), and held at 27 °C in a glass aquarium system containing ASW. Lighting was provided by one 1000 W, 14,000 K Hamilton Technology (Gardena, CA) metal halide bulb mounted 4 ft above the water surface and programmed to a 14h:10h light:dark cycle. Urchins were fed organic spinach daily and organic carrots 2-3 times per week.

Urchin spawning was initiated using 1-3 mL potassium chloride (0.5 M) injections into the coelom by inserting the needle through the peristomal membrane surrounding the mouth. Eggs were collected by inverting the female urchin over a beaker filled to the brim with ASW. The urchin aboral side was slightly submerged, so that the eggs were extruded directly into the seawater. After spawning was complete, the eggs were washed three times with an equal volume of fresh ASW and enumerated on a Sedgewick-Rafter counting chamber. Sperm was collected dry by aspiration with a micropipet tip and placed in a sterile 0.5 mL polypropylene Eppendorf tube. Sperm was kept chilled (not directly on ice) until used. Sperm was diluted 1:1000 in ASW to activate. The cell concentration was determined and motility was verified. Prior to beginning the assay, optimal fertilization rates (>95 %) were determined using four dilutions of sperm in a fertilization pre-test. Embryos (~200 in 50 µL volume) were placed in the glass vials containing 5 mL of sample. Artificial seawater and 4 mg/L sodium dodecyl sulfate (SDS) in ASW were included as assay controls. Vials were swirled gently to mix and the vial lids loosely attached to ensure adequate oxygenation during the course of the experiment. Embryos were incubated for 48 hours at 26 ± 0.5 °C under ambient lighting on a 12h:12h light:dark cycle.

Following incubation, an equal volume of 2X zinc-formalin fixative (Anatech, Poughkeepsie, NY) in ASW was added to each vial, and embryo developmental stage and developmental aberrations were scored, with a target of 100 embryos evaluated per sample replicate. Percent normal embryo development was calculated from the number of embryos reaching four-armed pluteus stage with no malformations, out of the total number of embryos in the vial. A one-way ANOVA with Dunnett's post-test was performed on the percent normal data using GraphPad Prism version 9.1.1 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

Sediments were collected at ten sites (Figure 3; Table 1) in the Bay for quantification of benthic foraminiferal populations. All of the sediment samples were dried at 80°C for 24 hours to ensure complete water loss and sub-samples were taken for subsequent percent total organic carbon (TOC), grain size, and FoRAM Index determination. For grain size analysis all sub-samples were wet-sieved (<63 µm) and oven dried at 50°C for 48 hours to determine the mud percent content (silt + clay) by weight difference. The coarser fractions (>63 µm) were dried sieved and represented as a phi (Φ) unit (-1= gravel; 0= very coarse sand; 1= coarse sand; 2= medium sand; 3= fine sand; 4= very fine sand; >4= mud) (Martínez-Colón et al., 2018). For the determination of TOC, the Loss-on-Ignition (LOI) method was implemented using a muffle furnace. Each sub-sample (1 g each) was oven dried at 105°C for 24 hours and after cooling to room temperature in a desiccator they were combusted at 550°C for 4 hours for TOC determination.

The coarser leftover material from the grain size analysis (-1 ≤ Φ ≤ 4) was homogenized and a 1 g sub-sample was collected for foraminiferal analysis following the Foram Index protocols (Hallock et a., 2003). The sampling of this study was done prior to the revised protocols suggested by Prazeres et al. (2020). No replicate sampling was done per site; the foraminiferal assessments and calculation of the FI were done in triplicate for each sub-sample. The FI calculation is based on Hallock et al. (2003):

FI = (10 x Ps) + (2 x Ph) + Po

where Ps = Ns/T, Ph = Nh/T, Po = No/T, T = total number of foraminiferal specimens counted, Ns

= is the number of "large benthic foraminifera" (LBF) counted, Nh is the number of "other heterotrophic" individuals counted, and No is the number of "stress tolerant" taxa counted (Prezeres et al., 2020). The FI values range from 1 to 10. Values <2 indicate "unsuitable" conditions for reef growth; values between 2-4 indicate "marginal" conditions for reef growth but likely "unsuitable" for recovery after a stress event; and values >4 are "conducive" for reef growth and recovery. The FI values of 3–5 can indicate that an area is undergoing environmental change (e.g., nutrification). The FI values reported are the calculated average of each sub-sample.

Results and Discussion

All chemistry data and metadata are available for public download via NOAA's National Centers for Environmental Information. (https://www.ncei.noaa.gov/access/metadata/landing-page/bin/iso?id=gov.noaa.nodc:0247462).

Water Sampling for Organics via CLAMs

CLAM units were successful at sampling a variety of contaminants; however, there were some issues with this new technology, most notably the failure of several units due to cracked housings (due to pressure at depths >6m). It should be noted that there was at least one 24 hour sample of each filter type at each site was collected. The smallest volume of water sampled was 68 L, which is far more than could be sampled with traditional discrete sampling.

Contaminants detected included current use pesticides, personal care products and pharmaceuticals, detected in very low concentrations (generally picogram per liter). Hydrocarbons were also detected at ng/L levels. Table 3a shows the contaminants which were detected and their maximum concentrations in the Bay. Figures 8 to 23 show the spatial distribution of the observed organic contaminants.

Spatially, multiple contaminants (e.g. aldrin, atrazine, chlordane) had their highest measured concentrations near the suspected freshwater anomaly. This would be consistent with a groundwater seep entering the Bay and bringing with it a variety of contaminants. Future work should consider sampling directly at this anomaly to confirm the pollutant vector.

Hydrocarbons (including PAHs) can be associated with the use and combustion of fossil fuels and other organic materials. Additional natural sources of PAHs can include decay of organic material (vegetation) and forest fires. The PAHs analyzed here are two to six ring aromatic compounds. PAHs can bioaccumulate in both aquatic and terrestri¬al organisms and many individual compounds are toxic. Some compounds such as benzo[a]pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k] fluoranthene, dibenzo[a,h]anthracene, and indeno[1,2,3-c,d]pyrene, are likely carcinogenic (ATSDR, 1995).

The majority of the non-hydrocarbon organic contaminants detected in the water column were pesticides, including many insecticides/insect repellants (aldrin, alpha-endosulfan, beta-endosulfan, oxy-chlordane, DEET, endrin ketone, heptachlor, mirex) and a few herbicides (atrazine, beta HCH, simazine). Hexachlorobenzene was the lone fungicide detected. Of the pharmaceutical compounds detected, only one (prednisolone) is a prescription medication. It is a corticosteroid used to treat a variety of conditions, and functions mainly as an anti-inflammatory. Multiple recreational, and in some cases illicit, drug-related compounds including: nicotine, cocaine and amphetamine were also detected.

Table 3a: Maximum observed concentrations for analytes detected via CLAM sampling in Fagatele Bay. Units are ng/L.

Hydrocarbons	Max. Concentration	Other Analytes	Max. Concentration
cis/trans Decalin	5.78	Aldrin	0.0005
C1-Decalins	2.49	alpha-Endosulphan	0.0094
C2-Decalins	2.52	Amphetamine	0.0993
C2-Fluorenes	14.49	Atrazine	0.0229
C3-Fluorenes	21.40	beta-Endosulphan	0.0044
Carbazole	2.05	Chlordane, oxy-	0.0082
C1-Phenanthrenes/ Anthracenes	17.11	Cocaine	0.0292
C1-Dibenzothiophenes	40.33	Cotinine	0.0107
C2-Dibenzothiophenes	14.33	DEET	0.1077
C3-Dibenzothiophenes	21.71	Desethylatrazine	0.0040
C2-Naphthobenzothiophenes	47.89	Endrin Ketone	0.0431
Perylene	51.28	HCH, beta	0.0020
Benzo(g,h,i)perylene	7.48	Heptachlor	0.0002
		Hexachlorobenzene	0.0215
		Mirex	0.0003
		Prednisolone	0.4037
		Simazine	0.0085

The demonstrated aquatic impacts of these pesticide and pharmaceutical compounds range from carcinogenic effects to endocrine disruption, although the effect of some compounds (e.g. cocaine) is not well described in the coral reef environment. Because of these deleterious environmental effects, many of these compounds have either been banned or restricted in use in the United States (CPEP 2006). Additionally, two metabolites of other compounds were detected: desethylatrazine (parent compound atrazine) and cotinine (parent compound nicotine).

Most of these analytes do not have environmental guidelines/thresholds above which sublethal ecological harm is expected, but mortality data (LC50) can be used for comparison. Table 3b shows the available LC50 information and the maximum observed values for those analytes. All analytes detected were orders of magnitude below published LC50 values. However, it is possible that sublethal or combinatory effects of multiple stressors could adversely affect organisms in the ecosystem. Even though these may be low concentrations, these data show that even in a relatively remote "pristine" system, a variety of waterborne contaminants are reaching the reefs and, if left unchecked, have the potential to adversely affect ecosystem health.

Table 3b: Comparison of maximum observed organic concentrations (via CLAM) with published LC50 values. Units are ng/L. All LC50 values are mortality endpoints from four day exposures to marine invertebrates, unless otherwise notes. From: https://cfpub.epa.gov/ecotox/search.cfm

Analytes	Fagatele Conc	LC50	Notes
Aldrin	0.0005	740	
alpha-Endosulphan	0.0094	30	
Amphetamine	0.0993	36310000	Freshwater for methamphetamine1
Atrazine	0.0229	48000	
beta-Endosulphan	0.0044	30	
Chlordane, oxy-	0.0082	11000	
Cocaine	0.0292	NA	
Cotinine	0.0107	NA	
DEET	0.1077	71000000	freshwater, fish

Table 3b continued.			
Desethylatrazine	0.0040	5100000	freshwater
Endrin Ketone	0.0431	37	
HCH, beta	0.0020	340	
Heptachlor	0.0002	30	
Hexachlorobenzene	0.0215	3300	1 day
Mirex	0.0003	56000000	Behavior (not mortality)
Prednisolone	0.4037	22290000	Freshwater; 1 day
Simazine	0.0085	3000000	fish

[1]From: http://actra.org.au/wp-content/uploads/2016/11/Mallavarapu-ACTRA-23-Sept-2016.pdf

Sediment Metals

In general, sediment metals concentrations (Figures 24-39) did not exceed previously published Sediment Quality Guidelines (SQG; Long et al. 1995) above which toxicity to benthic organisms might be expected. The exception to this was nickel, which exceeded the Effect Range Low (ERL, indicating possible sediment toxicity) at three sites (A,E,H Figure 3; yellow and orange dots on Figure 34), one of which (site E) also exceeded the Effects Range Median (ERM, indicating probable sediment toxicity). Metals concentrations, including nickel, likely represent a combination of natural (crustal erosion) sources and anthropogenic sources. Anthropogenic sources of nickel include metal plating and batteries, both of which could be present in the landfill. Elevated levels of nickel have been shown to have adverse effects on both marine invertebrates and fish, as well as coral larvae mortality (Novelli et al. 2003; Hunt et al., 2002; Goh, 1991).

Fagatele Bay had fewer sites with SQG exceedances for metals than recent studies in other locations on the island (Whitall et al. 2015; Mason and Whitall 2019). It is interesting to note that these other studies also documented high nickel sediment concentrations, which could be due to geologic sources of nickel island wide or improper disposal of nickel containing batteries (field team observation). Recent work in American Samoa has also suggested that changes in groundwater redox state, caused by increased nitrate loading and changes in dissolved inorganic carbon, may be increasing the solubility/mobility of metals in groundwater (Okuhata et al. 2020); this could also be influencing the metals concentrations of Fagatele Bay.

Nutrient Results

The nutrient data presented in this report are limited in time (one temporal data point per site) and do not capture the full picture of nutrient related water quality in Fagatele Bay. This study was not designed to fully characterize nutrient variability over time and these data should be viewed as a "snapshot" of ambient conditions that almost certainly change significantly with tides, currents and precipitation. The spatial variabilities (i.e. between sites) for the singular sampling timepoint for each analyte are shown in Figures 40 to 47.

Having acknowledged the limitations of these data, it is still useful to compare them to other nutrient data from the island to examine whether Fagatele Bay has atypical nutrient levels for the island. A previously published three-year dataset of nutrient data for Vatia Bay (north shore of the island of Tutuila, American Samoa) is a useful comparative dataset (Whitall et al. 2019).

Table 4 shows the mean and maximum values for each analyte for Vatia Bay, as well as the individual data points from Fagatele Bay. For most nutrient analytes, the Fagatele Bay values are similar to the mean values for Vatia, even though the two watersheds are quite different in terms of population: Vatia has a village (population of about 600 people) adjacent to the Bay, and inhabitants of Fagatele Bay watershed are fewer, more dispersed and farther from the Bay. Qualitative exceptions to the similarities between the two Bays are silica, which was lower in Fagatele than Vatia, and ammonium which is slightly higher in Fagatele. Lower silica in Fagatele is not surprising as there is significantly less stream flow (the primary source of silica, from crustal erosion) in that system compared to Vatia. It is unclear why ammonium values would be slightly higher in Fagatele, although there is some evidence of fecal pollution (see discussion under Colitag results). Interestingly, urea, which is also an indicator of fecal inputs is not high in this system. Additionally, data from this study can be compared to a time series dataset from the reef flat area of Fagatele (unpublished data, methods described in Comeros-Raynal et al. 2017). This study represents recurring (n=12) sampling of one site relatively near to the shore/waterfall. As Table 4 shows, the reef flat values are similar or perhaps slightly higher than what was

measured here. Additional work, including a recurring sampling effort, would be needed to make further assessments of the nutrient status of the Bay.

Table 4: Nutrient data from this study compared to two other studies on the island of Tutuila. Vatia data are from Whitall et al. (2019), and Fagatele Reef Flat data are unpublished data (methods described in Comeros-Raynal et al. 2017)

Site	NO_3-	HPO_4=	$HSIO_3$-	NH_4+	NO_2-	Urea	Total N	Total P
Site 1	0.006	0.012	0.157	0.023	0.001	0.002	0.355	0.024
Site 2	0.007	0.021	0.196	0.045	0.001	0.000	0.309	0.039
Site 3	0.014	0.010	0.190	0.038	0.001	0.001	0.325	0.038
Site 4	0.005	0.019	0.168	0.036	0.001	0.000	0.267	0.039
Site 5	0.003	0.020	0.192	0.056	0.001	0.001	0.347	0.039
Site 6	0.004	0.035	0.131	0.020	0.001	0.005	0.241	0.044
Site 7	0.002	0.020	0.135	0.024	0.001	0.000	0.342	0.034
Site 8	0.000	0.043	0.139	0.019	0.004	0.003	0.259	0.046
Vatia Mean	0.008	0.015	0.606	0.008	0.011	0.006	0.263	0.036
Vatia Max	0.311	0.052	14.286	0.167	0.145	0.028	1.399	0.258
Fagatele Reef Flat								
Mean	0.022			0.014	0.001			
Max	0.031			0.044	0.003			

Excess nutrients can adversely affect coral reef ecosystems in multiple ways. They can lead to macroalgal and benthic algal blooms, which can overgrow or outcompete the corals (Kuffner et al. 2006; Hughes and Tanner 2000; D'Angelo and Wiedenmann 2014). Additionally, excess nutrients can directly affect corals by reducing calcification and photosynthesis rates (Marubini and Davies, 1996), and by lowering fertilization and recruitment success (Harrison and Ward, 2001). Likely sources of excess nutrients in Fagatele Bay include human and animal waste (e.g. dogs, birds and bats), and chemical fertilizers.

Fecal Indicator Bacteria

All ten water samples were positive for total coliforms, with six samples testing positive for E. coli (Table 5, Figure 48). The freshwater samples from Sites W and T tested positive for coliforms and the main waterfall, Site W, was positive for E. coli. More than half (n = 6) of the Bay sample sites were positive for E. coli. Filter-sterilized bottled spring water used as the seawater diluent was negative for coliforms when tested with Colitag.

The presence of Escherichia coli (one species of coliform bacteria) in the water indicates fecal sources of bacteria (USEPA E. coli fact sheet, 2021). There were no obvious sources of fecal pollution around the Bay but E. coli could originate from livestock, pets, wildlife or humans. Bats are common on the island and have been found to harbor fecal coliforms and pathogens (Banksar, 2016). Dogs roam the island freely, and various bird species were observed in the watershed. Future visual inspection and sampling along the segment of the stream leading to the waterfall would aid location and identification of potential sources of fecal pollution. There was no definitive spatial pattern for the presence or absence of E. coli at the sample sites. Additional sampling in transects could help locate potential sources of fecal contamination. Animal sources of fecal pollution could be identified with microbial source tracking (MST). Source tracking with quantitative polymerase chain reaction (qPCR) has the potential to identify the host as human, ruminant (e.g. cow), avian, swine or canine (Vadde, 2019; Stewart, 2013) or by analyzing for fecal sterols and stanol ratios (Emrich et al., 2017).

Colitag tests provided presence/absence results. Although some tests turned positive fairly quickly, the concentrations of coliforms and E. coli were not determined, meaning that these data are not directly applicable to evaluating possible exceedances of water quality standards for Fecal Indicator Bacteria (FIB). For this type of application, a different methodology, e.g. direct counts from membrane filtration methods for fecal coliforms (mFC media), E. coli (mTEC) and/or enterococcus (mEI), would be required. For this project, only one set of samples (one timepoint) was tested for coliforms, representing a snapshot of microbial water quality. Repeated sampling would capture a more accurate assessment of the coliform and E. coli levels in Fagatele Bay. Previous to this study, FIB were not regularly monitored and the positive results were unexpected.

Table 5. Coliform results for Fagatele Bay, National Marine Sanctuary, American Samoa using Colitag. Total coliforms were present in all samples. A bacterial indicator of fecal pollution, E. coli, was present in six of the ten samples, including the waterfall and five sample sites.

Sample Site #	Sample Description	Total Coliforms (Positive/Negative)	E. coli (Positive/Negative)
W	Waterfall	+	+
T	waterfall trickle	+	-
1	Site 1	+	+
2	Site 2	+	+
3	Site 3	+	-
4	Site 4	+	+
5	Site 5	+	-
6	Site 6	+	-
7	Site 7	+	+
8	Site 8	+	+
9	Anomaly	NA	NA
Negative control	Filter-sterilized bottled spring water	-	-

Mutagenicity Screening of Water Column

No mutagenic activity was detected in any of the sample extracts using Ames tester strains TA98 or TA100 with or without metabolic activation at 1000x ambient concentration (Figure 49). This increased (above ambient) test concentration was used as a conservative screening analysis; had mutagenicity been detected, additional (lower) test levels would have been used as well. The negative controls had a few spontaneous revertants, considered background for these strains. As expected, the positive control for TA98 without S9 metabolic activation performed accurately with over two-fold the number of background colonies as a result of exposure to the appropriate chemical mutagen for each strain/S9 combination.

Many compounds can affect the health of corals. If mutagenic chemical compounds are found, they can trigger legal/regulatory action. The Ames test is a standard method that can be used to screen for mutagenic compounds in the water surrounding coral reefs. None of the samples tested as mutagenic with the Ames test in this study (Figure 49). The sample results for TA100 without S9 activation are considered presumptively negative because the positive control chemical, sodium azide, was not available at the time of testing. The lack of detected mutagenicity may be due to the relatively low levels of chemicals such as pharmaceuticals and personal care products (PPCPs) found in the Bay (Table 3a) and the results could be influenced by the strains of tester bacteria used. Each strain has a particular mutation that can identify the type of mutagenicity, such as oxidative or sensitivity to aldehydes, which causes the sensitivity of each strain to vary. Negative and positive controls are needed for each strain and testing factor (S9+ and S9-). The positive controls for three out of the four strain/S9 combinations were positive, as expected. The sample results for TA100 without S9 activation are considered presumptively negative because the positive control chemical, sodium azide, was not available for use at the time of testing. Future work could include other related methodologies; researchers have found varying sensitivities to various TA strains which may provide additional data. Standard S9 liver enzymes are produced by exposing male mice to Arochlor-1254, a known mutagen (Maron and Ames, 1983). Alternatively, an S9 mix made from golden Syrian hamster liver exposed to Arochlor-1254 is commercially available and less commonly used. Hakura, et al (2002)

conducted the Ames test using enzymes made from human liver. If mutagenicity is detected, action can be taken to identify chemical compounds and mitigate their sources.

Water Quality Analyses for Sea Urchin Bioassay

Water quality measurements were conducted on all test samples for the sea urchin embryo toxicity test. The measured parameters were all within acceptable test ranges for the bioassay (Table 6; Carr et al. 2006). Salinity ranged from 36-37 psu and pH ranged from 8.25-8.2. Ammonia nitrogen was below the limit of detection for all samples. Slightly elevated salinity (+1 psu) for the filtered samples was likely due to salt accumulation on the HLB columns following filtration.

Table 6. Water quality results for control and Fagatele Bay diluted sample eluates. BLD = below limits of detection (0.006 mg/L)

Sample	Salinity (psu)	pH	TAN	UAN
			(mg/L)	(µg/L)
Site 1	37	8.26	BLD	BLD
Site 2	37	8.26	BLD	BLD
Site 3	37	8.27	BLD	BLD
Site 4	37	8.26	BLD	BLD
Site 5	37	8.25	BLD	BLD
Site 6	37	8.25	BLD	BLD
Site 7	37	8.27	BLD	BLD
Site 8	37	8.25	BLD	BLD
Freshwater Anomaly	37	8.26	BLD	BLD
Solvent control	37	8.27	BLD	BLD
ASW	36	8.26	BLD	BLD
SDS	36	8.27	BLD	BLD

Sea Urchin Embryo Development Toxicity Assay

The sea urchin embryo development assay was used to evaluate the effects of contaminants captured on the HLB columns. It should be noted that not all contaminants will be captured by the HLB columns, so these results are only based on the contaminants present in the eluate.

As expected, embryos incubated in the SDS positive control exhibited significantly (ANOVA, $p < 0.0001$) delayed development after 48 hours (Figure 50). Both solvent and ASW controls resulted in similar embryo development. Fagatele Bay reconstituted samples exhibited no toxicity (i.e., toxicity being defined as significantly decreased normal development compared to the ASW control) (Figure 50). For reference, normal L. variegatus development is presented in Figure 51 and representative images from embryos incubated in reconstituted sample eluates are shown in Figure 52.

While not significant, higher frequencies (>2x compared to the ASW control, mean = 4.3 %) of underdeveloped embryos were observed for samples from site 3 (mean = 11.8 %), site 7 (mean = 15.3 %), site 8 (mean = 8.8 %) and site 9 (temperature/turbidity anomaly, mean = 12.8 %), indicating possible low-level impacts in these areas (Figure 53). Toxicants which can result in slowed or arrested development for sea urchin embryos include detergents, such as SDS (Bellas et al., 2005), sodium hypochlorite (Rock et al., 2011), crude oil (Hamdoun et al., 2002), pesticides (Manzo et al. 2006; Perina et al. 2011) and metals (Cu, Pb, Se, Ni, Ag, Zn) (Bielmyer et al., 2005; Rouchon and Phillips 2016). The pesticides and metals studies noted that embryo development is correlated to dose, with an increase in developmental delay linked to higher concentrations.

Foram Index

The characteristics of the sediment sampled for forams was quite coarse; for grain size, gravel is the most abundant (Φ = -1) median grain (see Table 7).

Table 7: Foram Index results by site. Median grain size (Φ). See discussion in text regarding sample size caveats.

Site #	Mud (%)	Φ	Foram Index
1	0.1	-1	1.0
2	0.0	-1	0.0
4	0.0	-1	1.7
5	0.0	-1	1.8
6	6.4	-1	5.5
7	0.1	-1	6.6
8	0.0	-1	0.0
A	2.0	-1	
C	0.2	-1	2.4
K	0.1	-1	4.3

A total of 83 benthic foraminifers were counted amongst the 10 sediment samples with 20 generic groups being identified (Table 8). Given the very low values of individuals counted (0-26) in a sample, the diversity as well as the density of the foraminifers was very low and highly uncharacteristic of a coral reef setting. These sample sizes are below the minimum recommended sample size (>50 individuals per gram of sediment) found in Prazeres et al. (2020). According to these guidelines, the FI values in this study may not be valid. They are shared here for qualitative assessment and discussion.

Seven out of the ten stations had FI <2 which is indicative of “unsuitable” reef conditions (Figure 54). Only three stations (D, I, and K) had values >4 which indicates that the environment is “conducive” for reef development. It seems that the FI values are artifacts of two potential factors given that the reef is in relatively healthy condition. First, the sampling sites were not optimized for foraminifera collections, i.e. they were co-located with other collections or selected based on available substrate. Second, the sediment texture may have been a confounding factor; most of the sediment samples (eight out of ten) were very angular, jagged, coarse grained, and large benthic foraminifers like Amphistegina were broken. This strongly suggests that the wave energy is very high in the study area, which may have affected the foraminfera at these sites. A similar situation with the FI is observed in the small fringing coral reefs of Jobos Bay in Puerto Rico were a difference of 50 feet of water depth is observed in a narrow area between the reef front (coarse grained sediments) and forereef (carbonate mud).

Conclusions

This study used seven different water quality assessment methods in a preponderance of evidence approach to assess the pollution status of Fagatele Bay.

Key findings included:

1) CLAM in situ active monitoring devices were effective in quantifying aqueous organic contamination of the Bay. Thirty-two organic contaminants, including hydrocarbons, pharmaceuticals and agrochemicals were detected, although concentrations were too low to be of likely ecological impact.

2) Sediment concentrations of metals were similar to other sites on the island; nickel was elevated above SQG at three sites which is likely due to a mix of natural (crustal erosion) and anthropogenic (landfill) sources.

3) A snapshot of bottom water nutrient concentrations suggests that the Bay is similar to or possibly less impacted by nutrients than other systems on the island. However, this data set is temporally limited (n=1) and should be caveated as such.

Table 8: Foram genus results by site. Green= symbiont bearing; yellow= other heterotrophic; red=stress tolerant.

Genus name	1	2	4	5	6	7	8	A	C	K
Amphistegina	0	0	0	0	1	1	0	0	0	2
Anomalinoides	0	0	0	1	0	0	0	0	0	0
Asterigerina	0	0	0	0	0	0	0	0	0	1
Calcarina	0	0	0	0	0	1	0	0	0	0
Cibicidoides	0	0	0	2	0	1	0	9	0	0
Discorbis	0	0	0	0	0	0	0	5	0	3
Elphidium	0	0	3	0	0	0	0	0	4	0
Elphidiella	0	0	0	0	0	1	0	0	1	0
Heterostegina	0	0	0	0	0	1	0	0	0	0
Miliolinella	0	0	1	0	0	0	0	0	0	0
Nonion	0	0	0	1	0	0	0	3	1	0
Planorbulina	0	0	0	0	0	0	0	2	6	0
Pseudohauerina	0	0	0	0	0	0	0	0	0	1
Pyrgo	0	0	2	0	0	0	0	0	0	0
Quinqueloculina	0	0	2	0	0	0	0	2	0	0
Siphogenerina	0	0	0	0	0	0	0	1	2	0
Spiroloculina	0	0	0	0	0	0	0	1	4	1
Sortites	0	0	0	0	0	0	0	0	2	0
Textularia	2	0	0	0	1	0	0	1	0	1
Triloculina	0	0	3	0	0	0	0	2	3	1
Total Count	2	0	11	4	2	5	0	26	23	10
FI	1.0	0.0	1.7	1.8	5.5	6.6	0.0	1.8	2.4	4.3

4) All water samples (n=10) tested positive for coliform and the majority (six) tested positive for E. coli, which is indicative of a potential issue with fecal pollution from mammalian or avian waste.

5) The Ames test (for mutagenicity) was negative for all samples even at concentrations 1000x ambient, suggesting that, based on this methodology, there is no risk of mutagenicity from any individual or combined pollutants.

6) No significant toxicity was observed in the sea urchin bioassays from HLB-eluted samples from throughout the Bay. Several sites however did display increased developmental anomalies, though did not reach statistical significance from controls. Overall these results suggest that the suite of compounds captured from Bay waters onto HLB columns did not present a threat to ecosystem health at their current concentrations.

7) The inconsistent results of the Foram Index were confounded by extremely coarse substrate due to high wave energy which resulted in very low foraminiferal counts.

Overall, this preponderance of evidence approach did not find any indication of significant degradation of the water quality of the Bay. The only contaminants of concern identified in this study could be nickel which is elevated above SQG at three sediment sites, and fecal pollution (coliform and/or E. coli were detected in all samples). However, because anthropogenic pollutants are reaching this valuable marine resource, additional monitoring and assessment work may be warranted.

Our scientific recommendations include:

1) Additional sampling/analysis with methodologies beyond those used in this study to determine extent and sources of fecal pollution (bacterial pathogens and associated nutrients);

2) Future stream, groundwater and/or soil sampling to determine source of elevated sediment nickel concentrations;

3) Periodic (e.g. every three to five years) reassessment of pollution condition at a subset of sites to ensure that the low levels of pollution currently in the Bay are not increasing.

4) Implement groundwater well sampling to determine contaminant concentrations in groundwater as a potential vector of pollution to the Bay.

5) Investigate the potential impact of the landfill on other nearby systems (e.g. Fagalua/Fogoma).

These data have been used by the National Marine Sanctuary as part of the Sanctuary assessment (Condition Report) process; this report is in review and has an anticipated release date of 2022. Additionally, these data will be useful to coastal managers for tracking future changes to the system. These unique data are foundational to understanding land-based sources of pollution in Fagatele Bay unit of the Sanctuary.

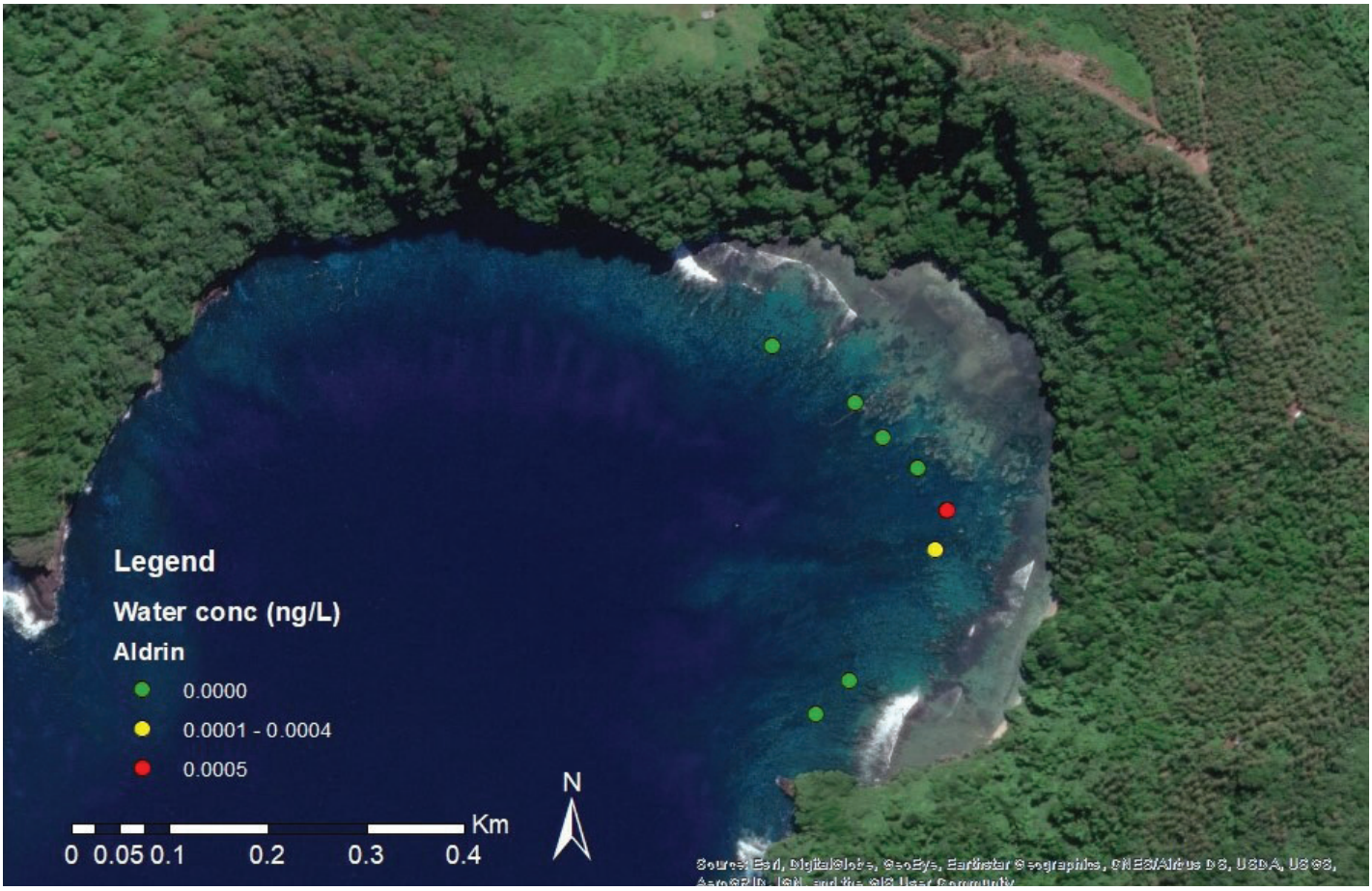

Figure 8: Water concentrations (CLAM derived) of aldrin in Fagatele Bay.

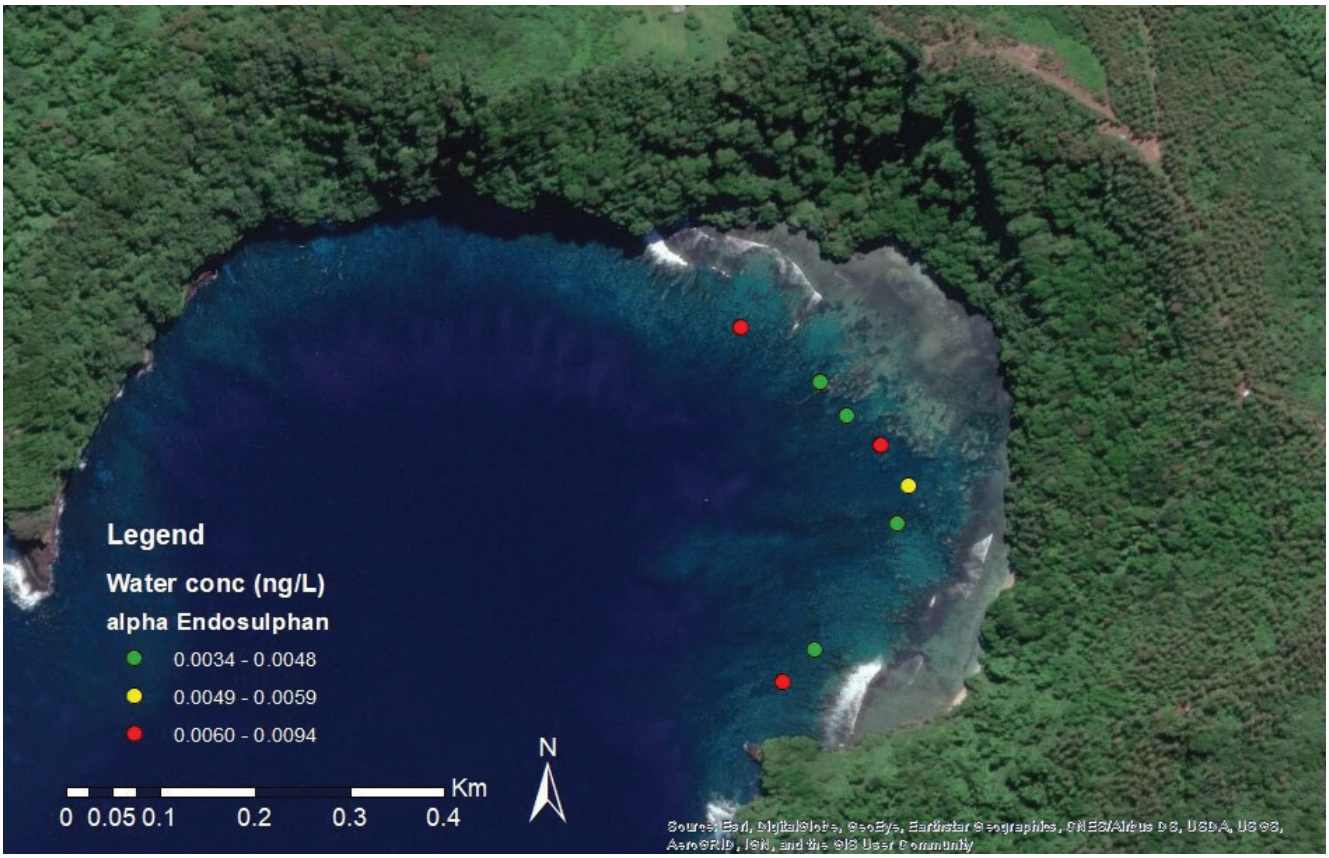

Figure 9: Water concentrations (CLAM derived) of alpha endosulfan in Fagatele Bay.

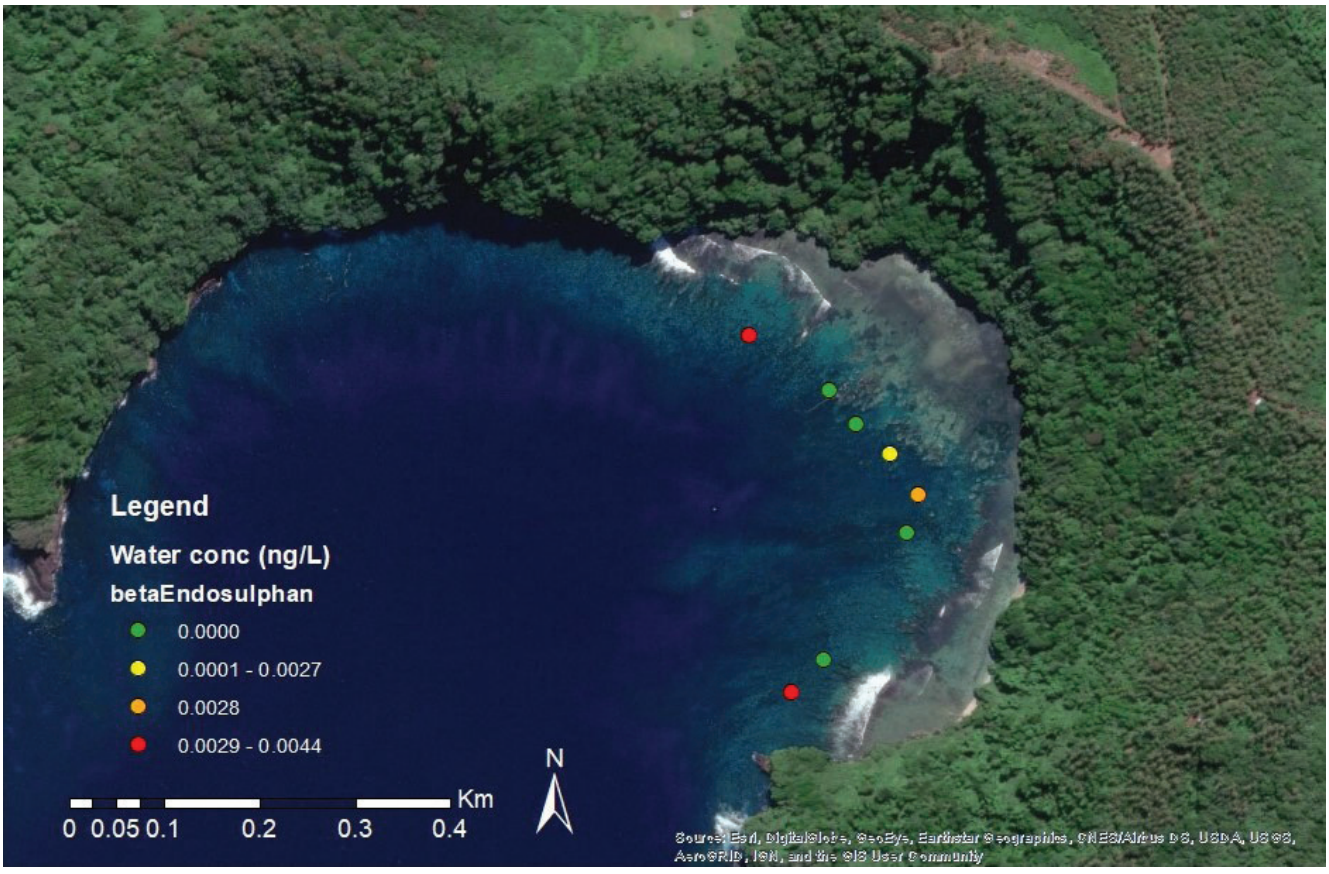

Figure 10: Water concentrations (CLAM derived) of beta endosulfan in Fagatele Bay.

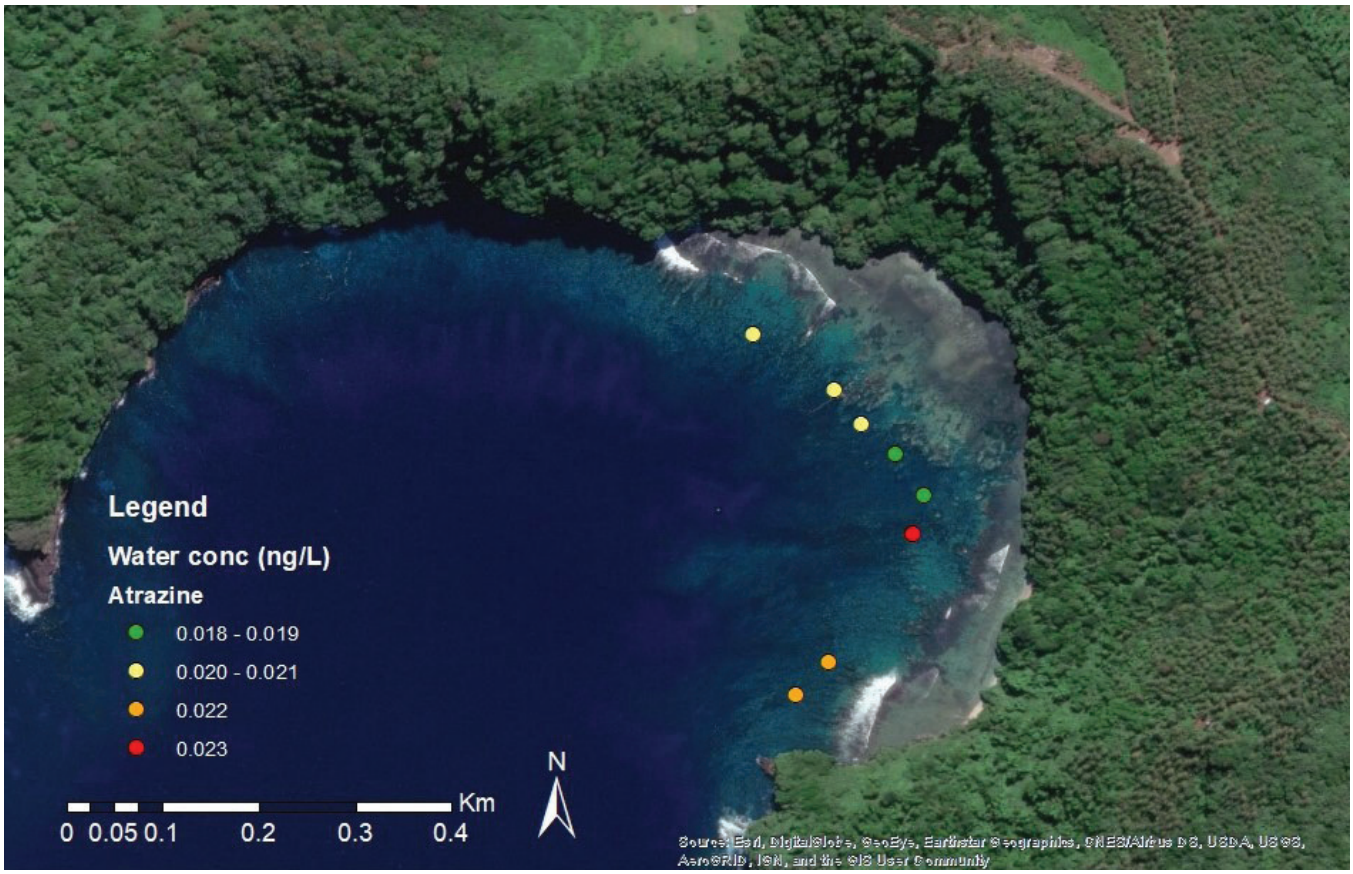

Figure 11: Water concentrations (CLAM derived) of atrazine in Fagatele Bay.

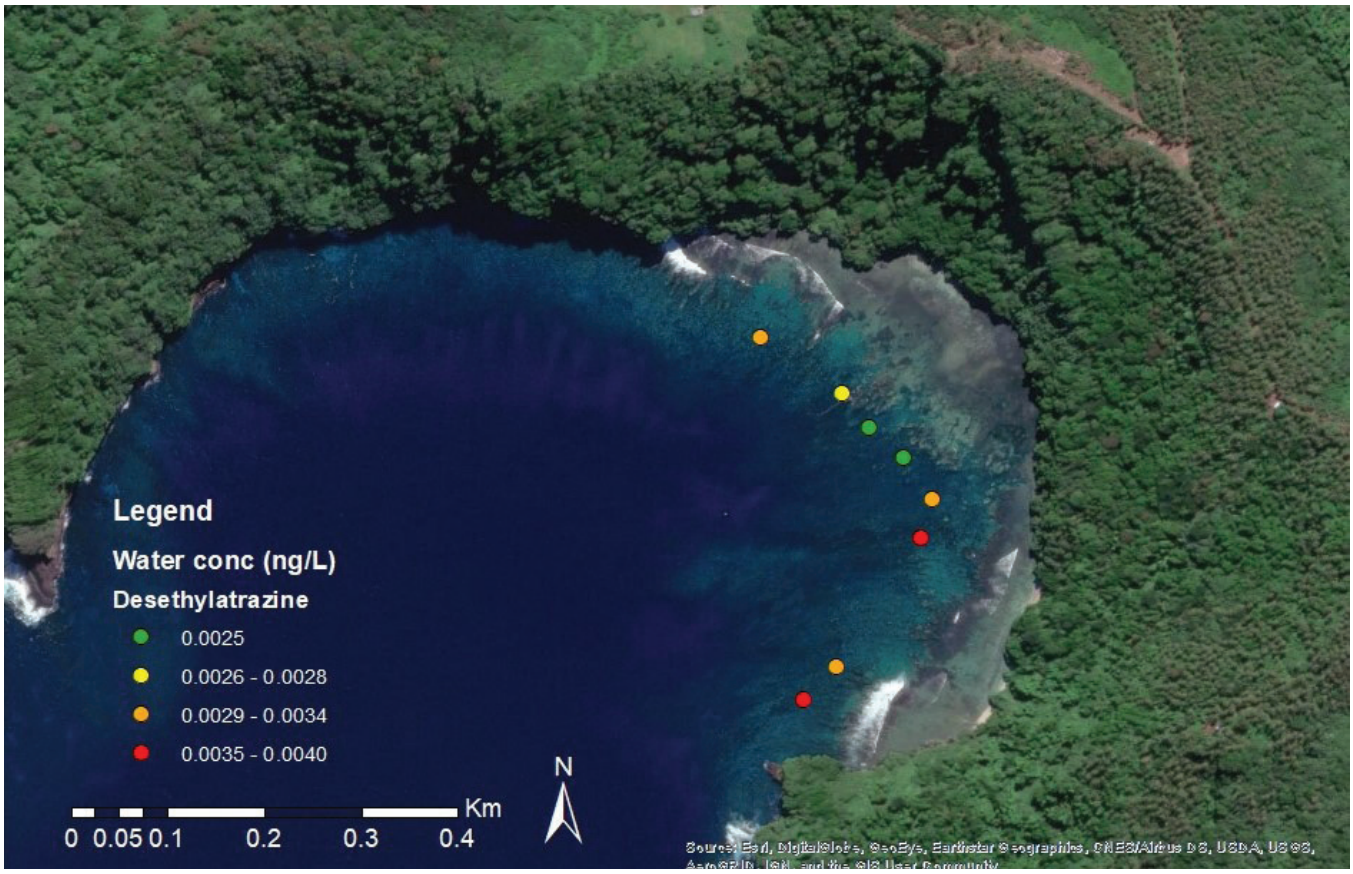

Figure 12: Water concentrations (CLAM derived) of desethylatrazine in

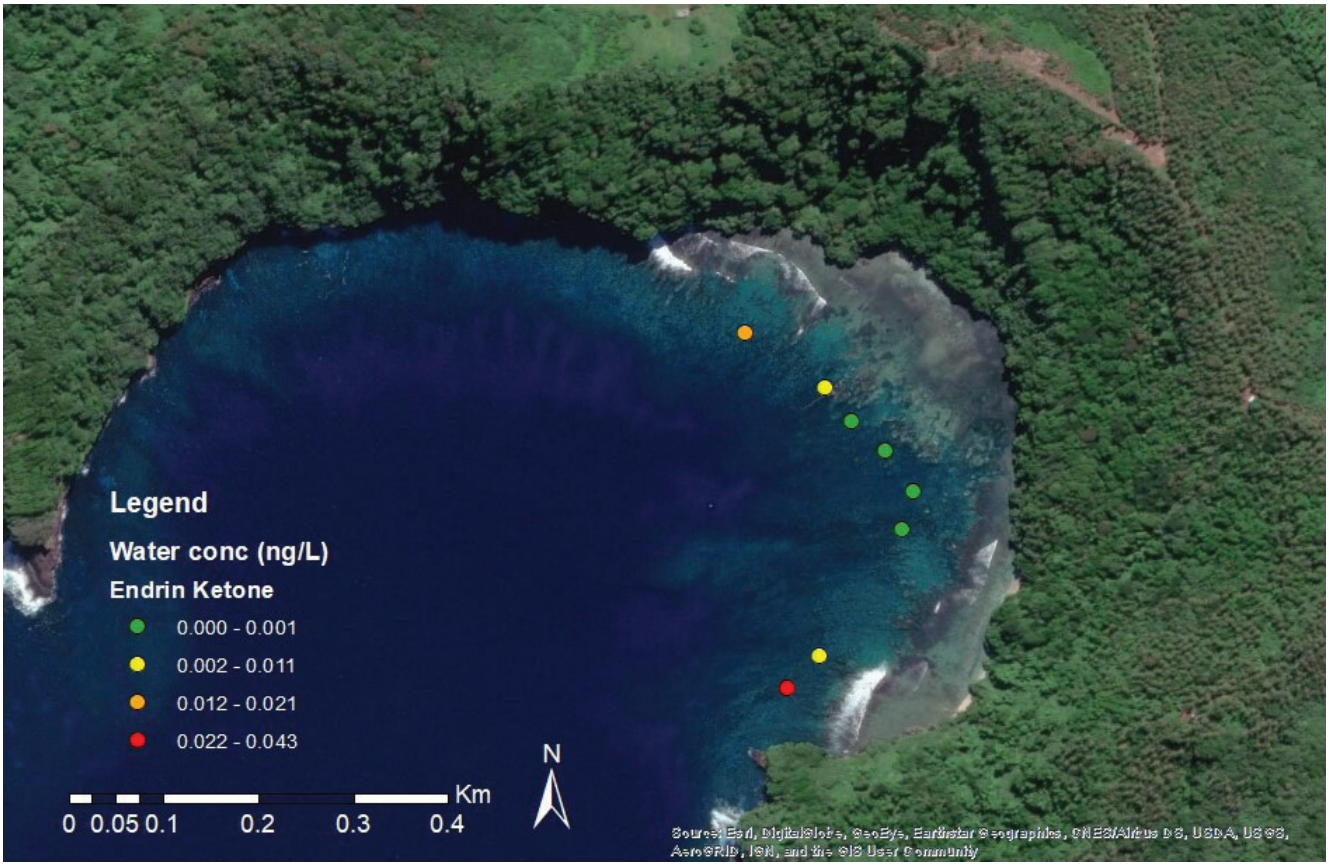

Figure 13: Water concentrations (CLAM derived) of endrin ketone in Fagatele Bay.

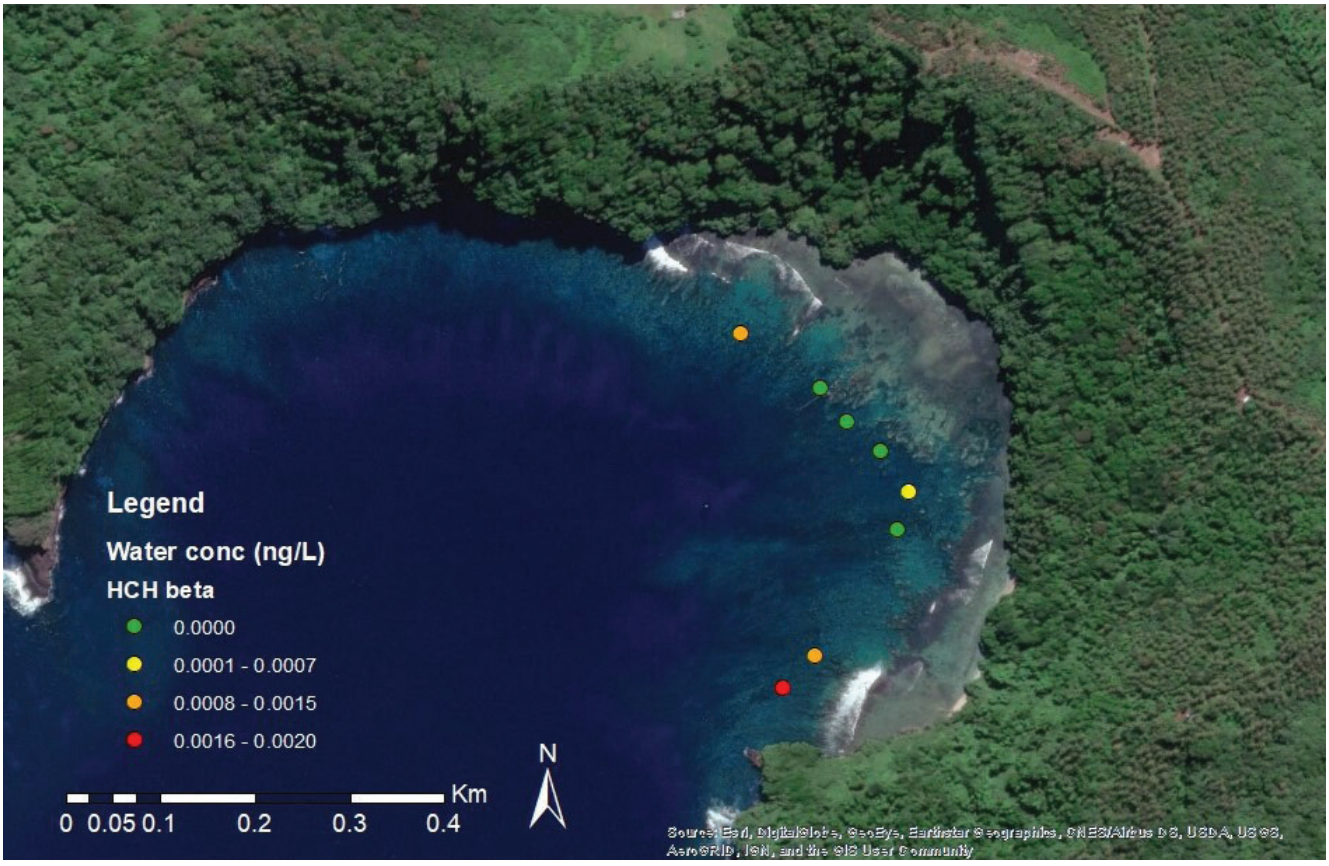

Figure 14: Water concentrations (CLAM derived) of Hexachlorocyclohexane (HCH) beta in Fagatele Bay.

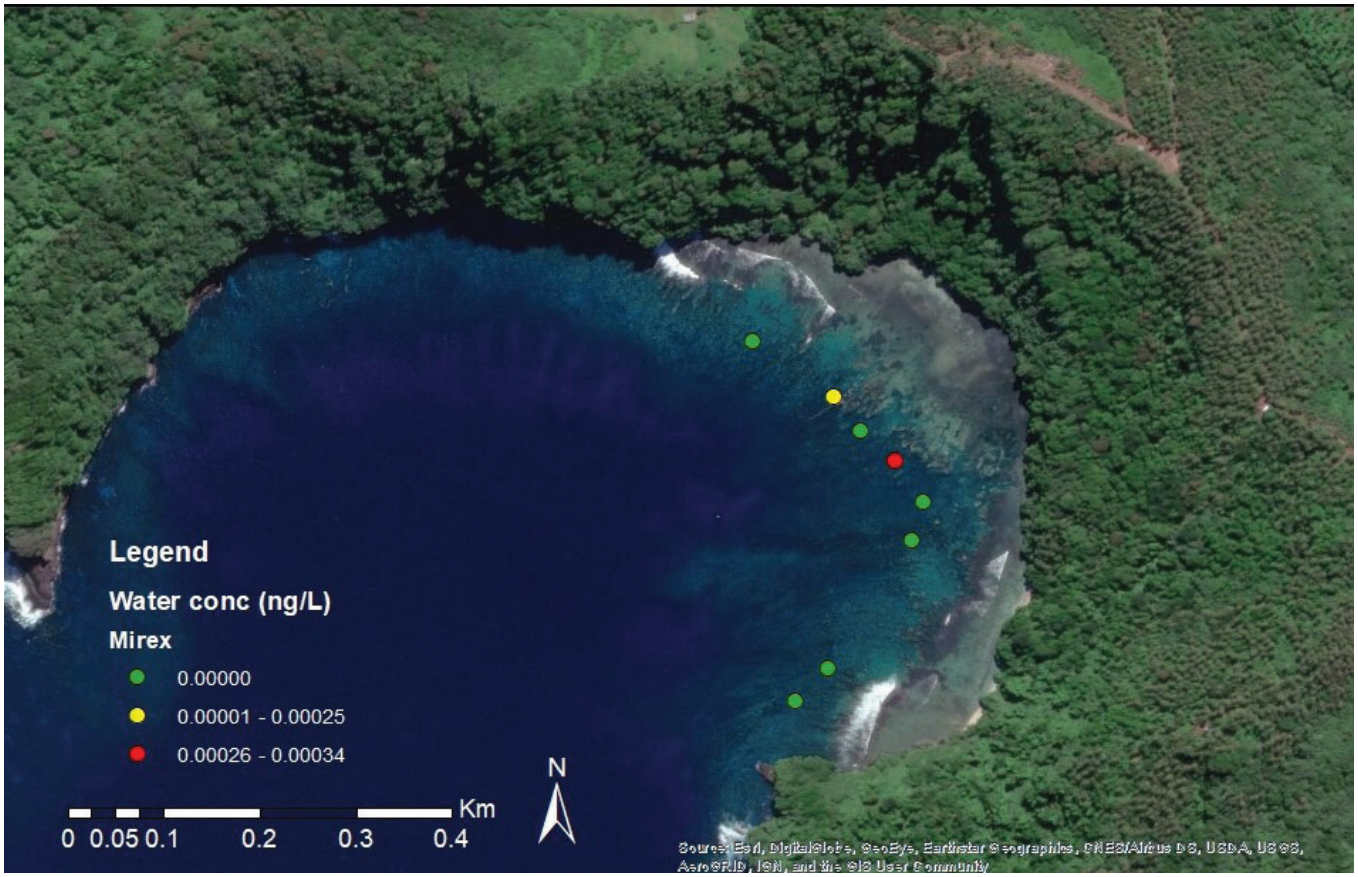

Figure 15: Water concentrations (CLAM derived) of mirex in Fagatele Bay.

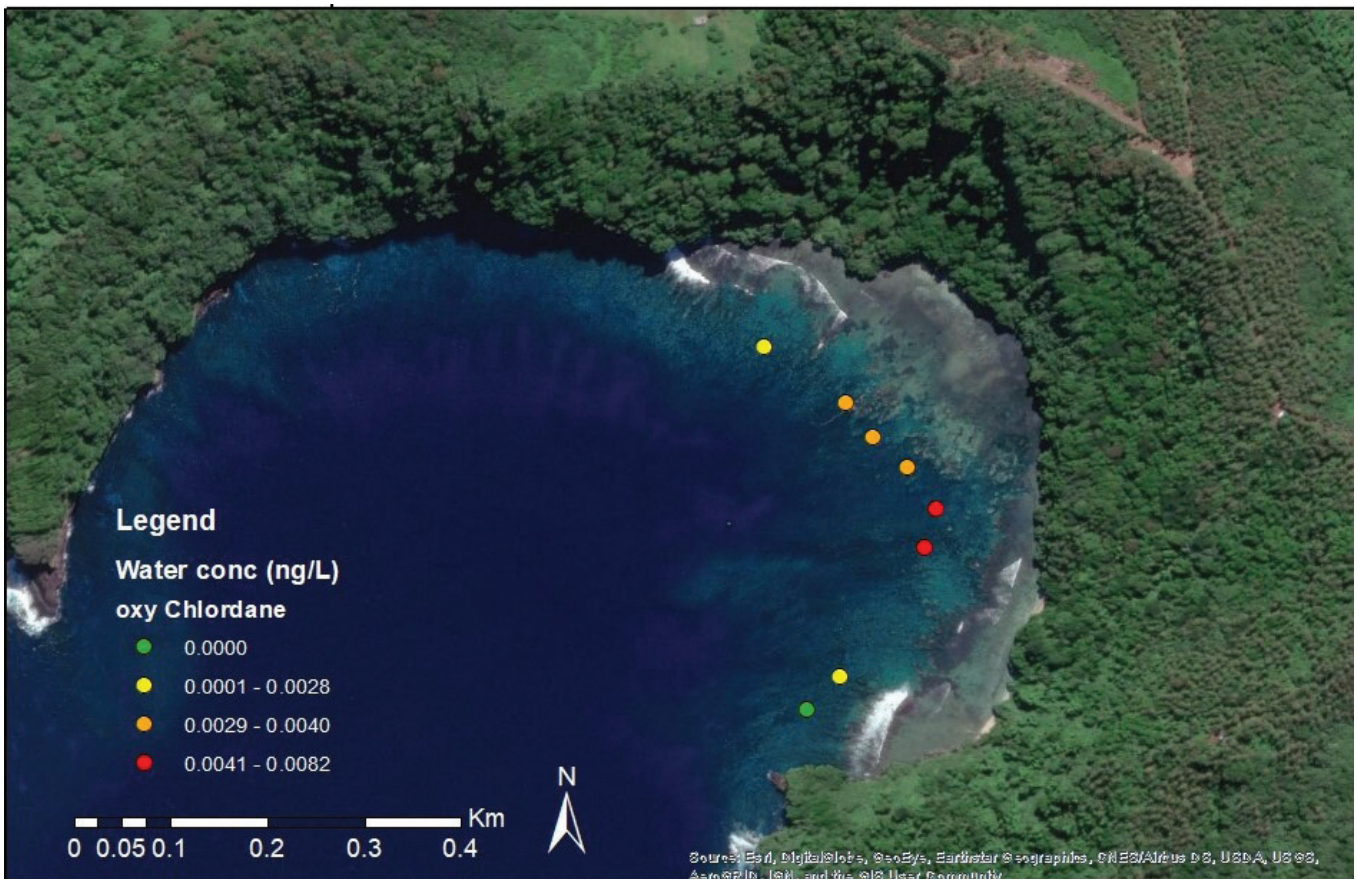

Figure 16: Water concentrations (CLAM derived) of oxychlordane in Fagatele Bay.

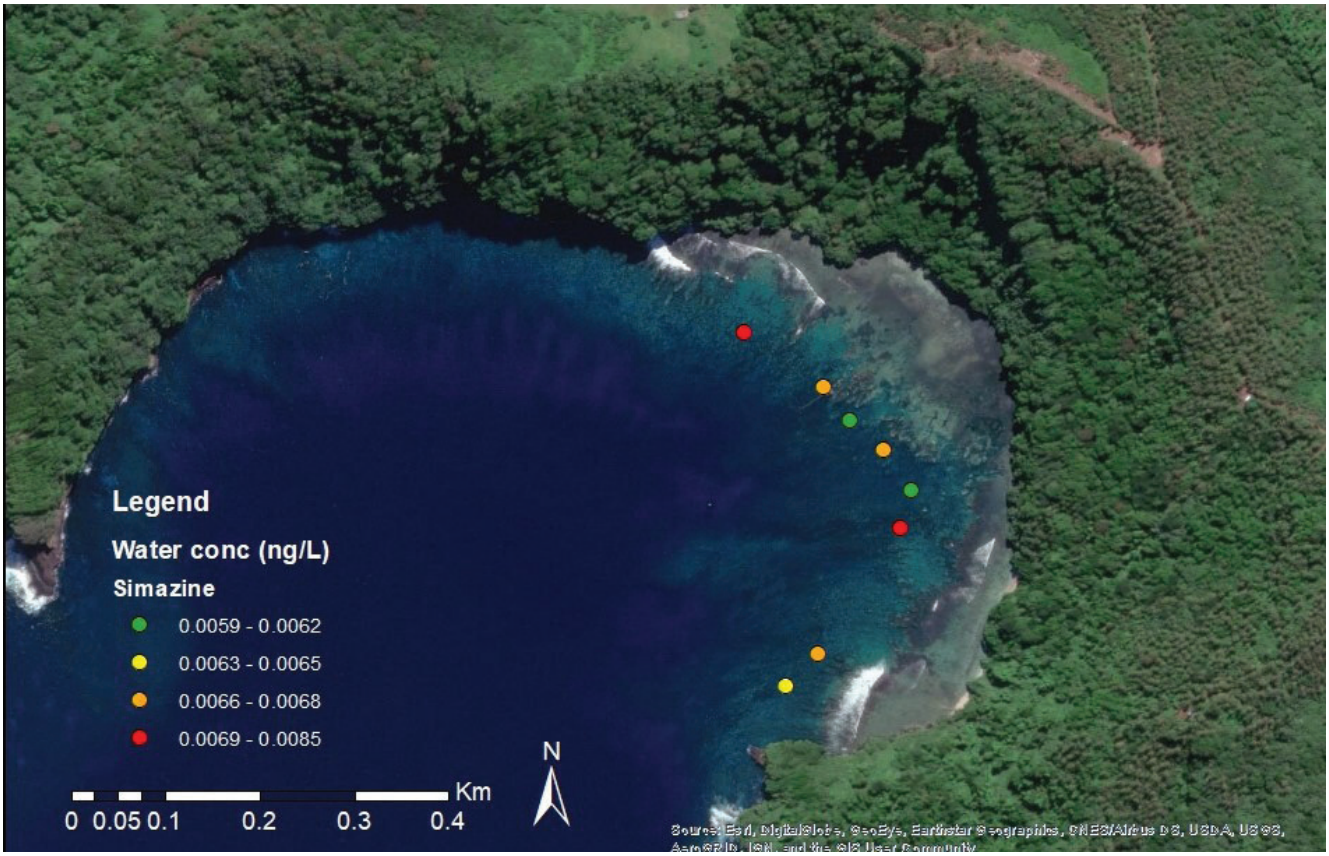

Figure 17: Water concentrations (CLAM derived) of simazine in Fagatele Bay.

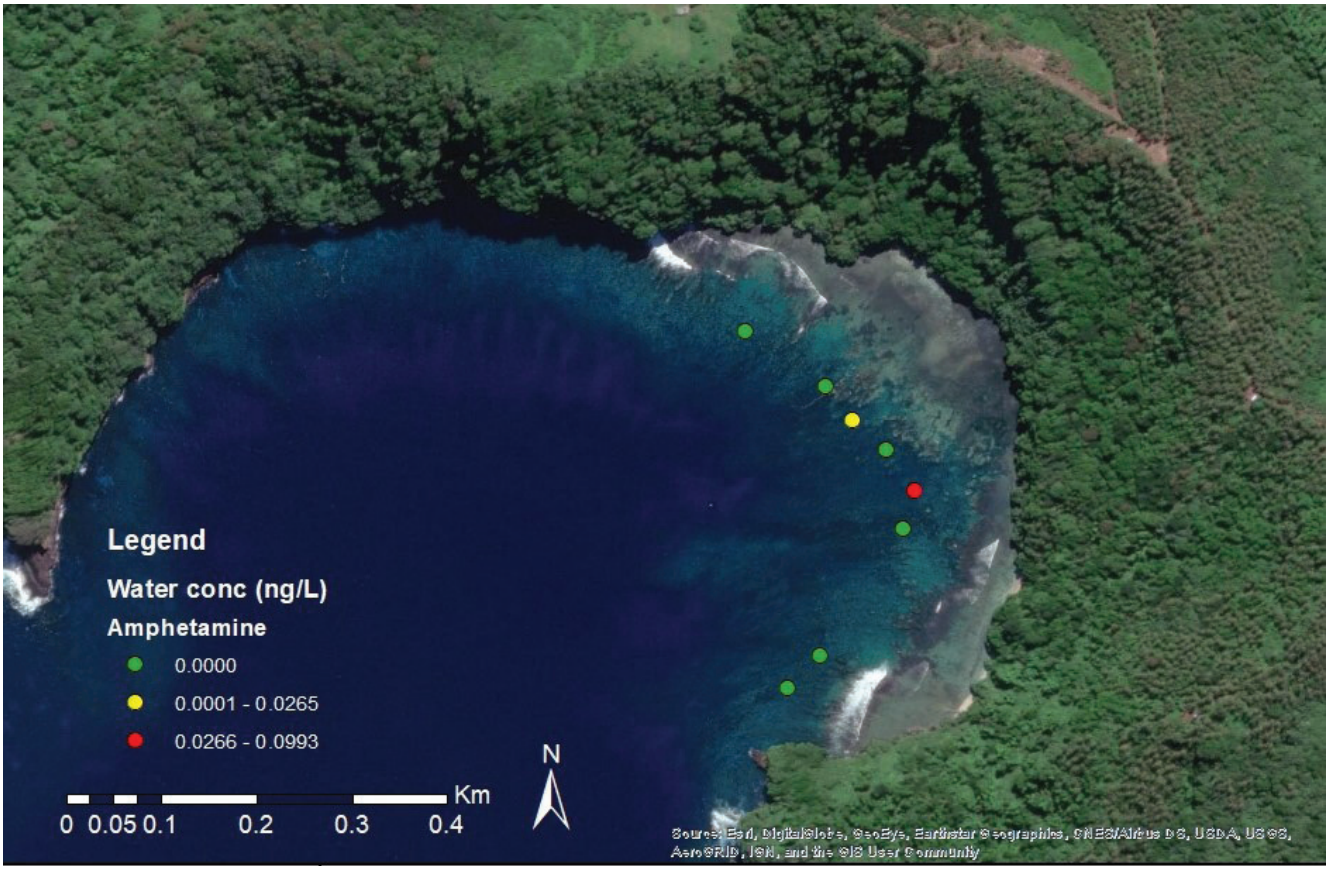

Figure 18: Water concentrations (CLAM derived) of amphetamine in Fagatele Bay.

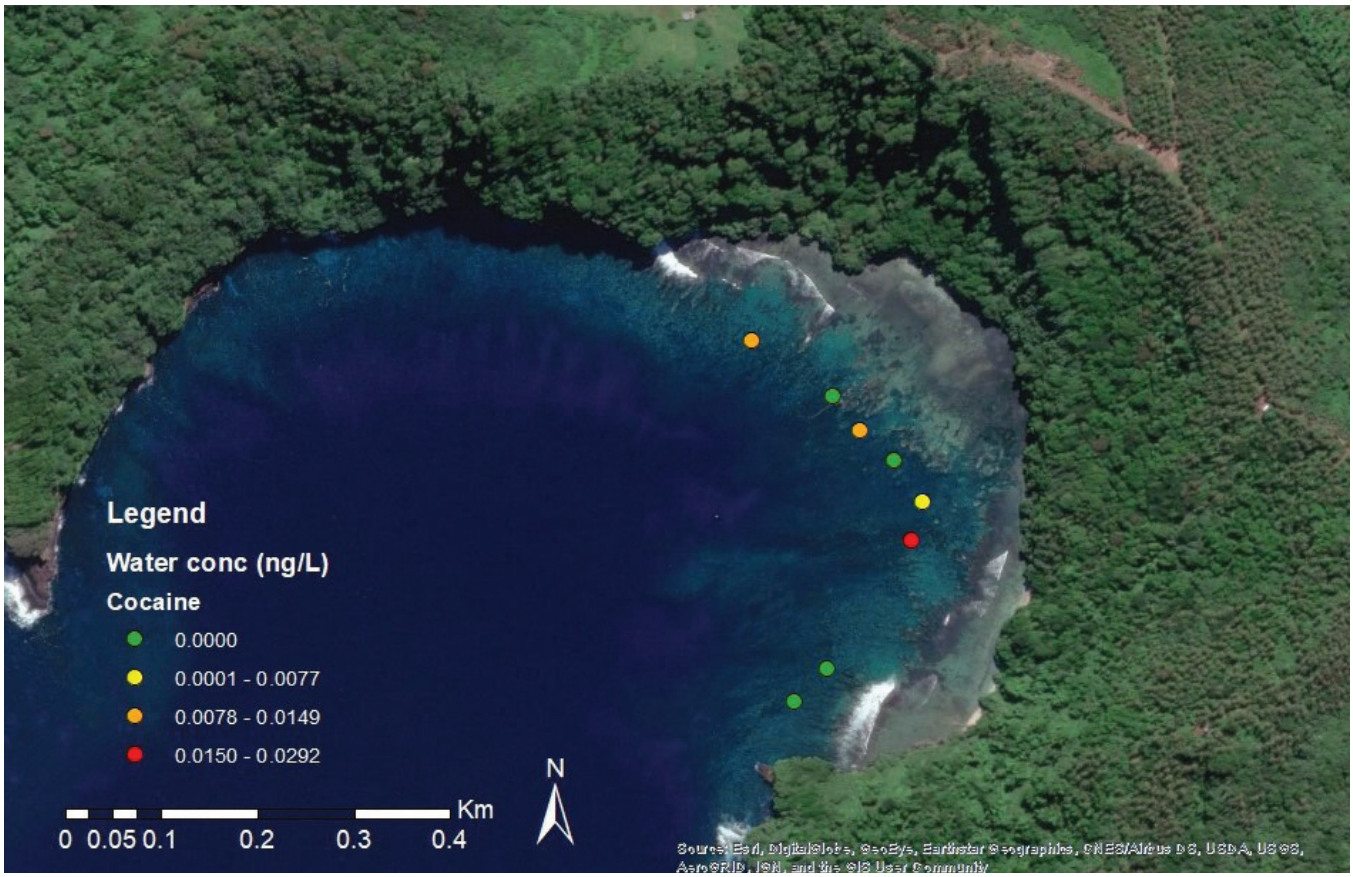

Figure 19: Water concentrations (CLAM derived) of cocaine in Fagatele Bay.

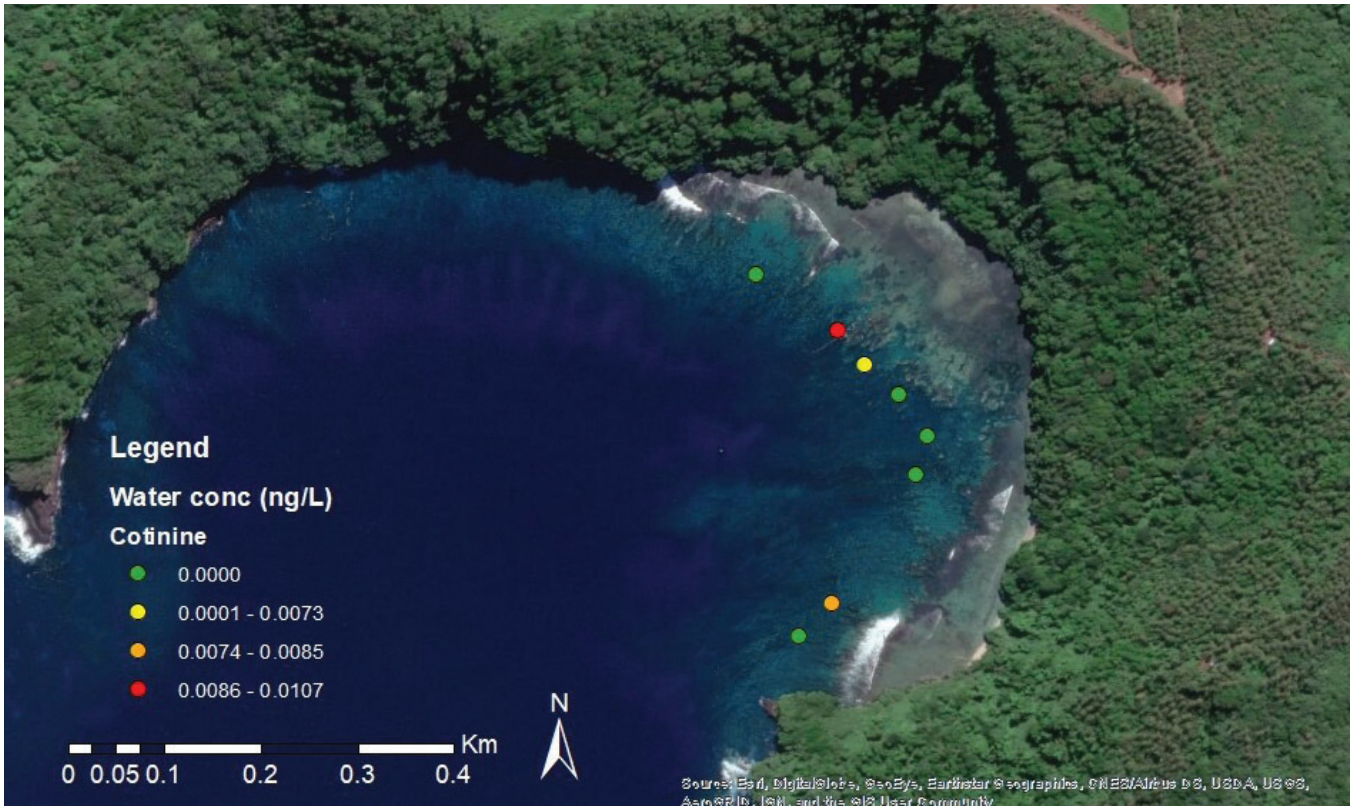

Figure 20: Water concentrations (CLAM derived) of cotinine in Fagatele Bay.

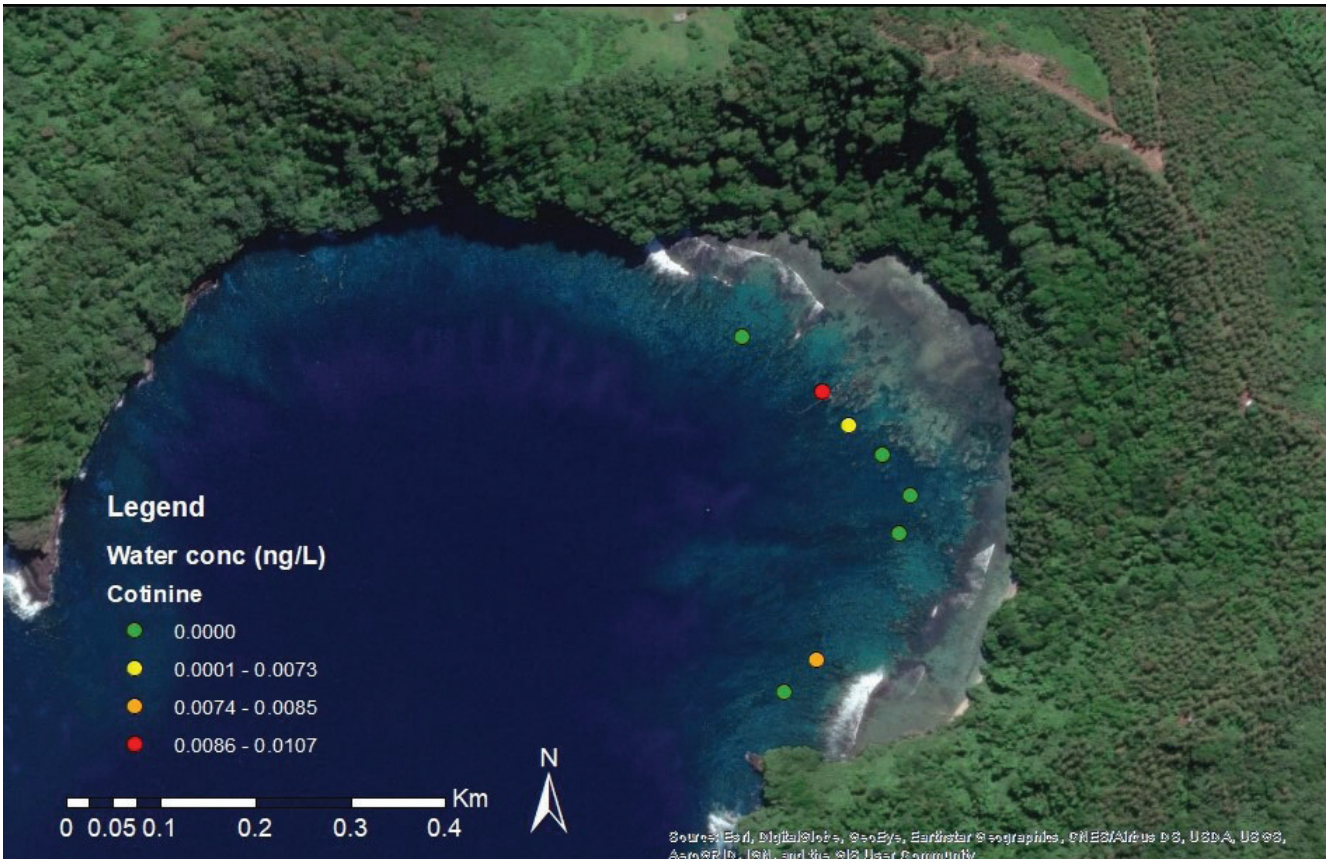

Figure 21: Water concentrations (CLAM derived) of N,N-diethyl-meta-toluamide (DEET) in Fagatele Bay.

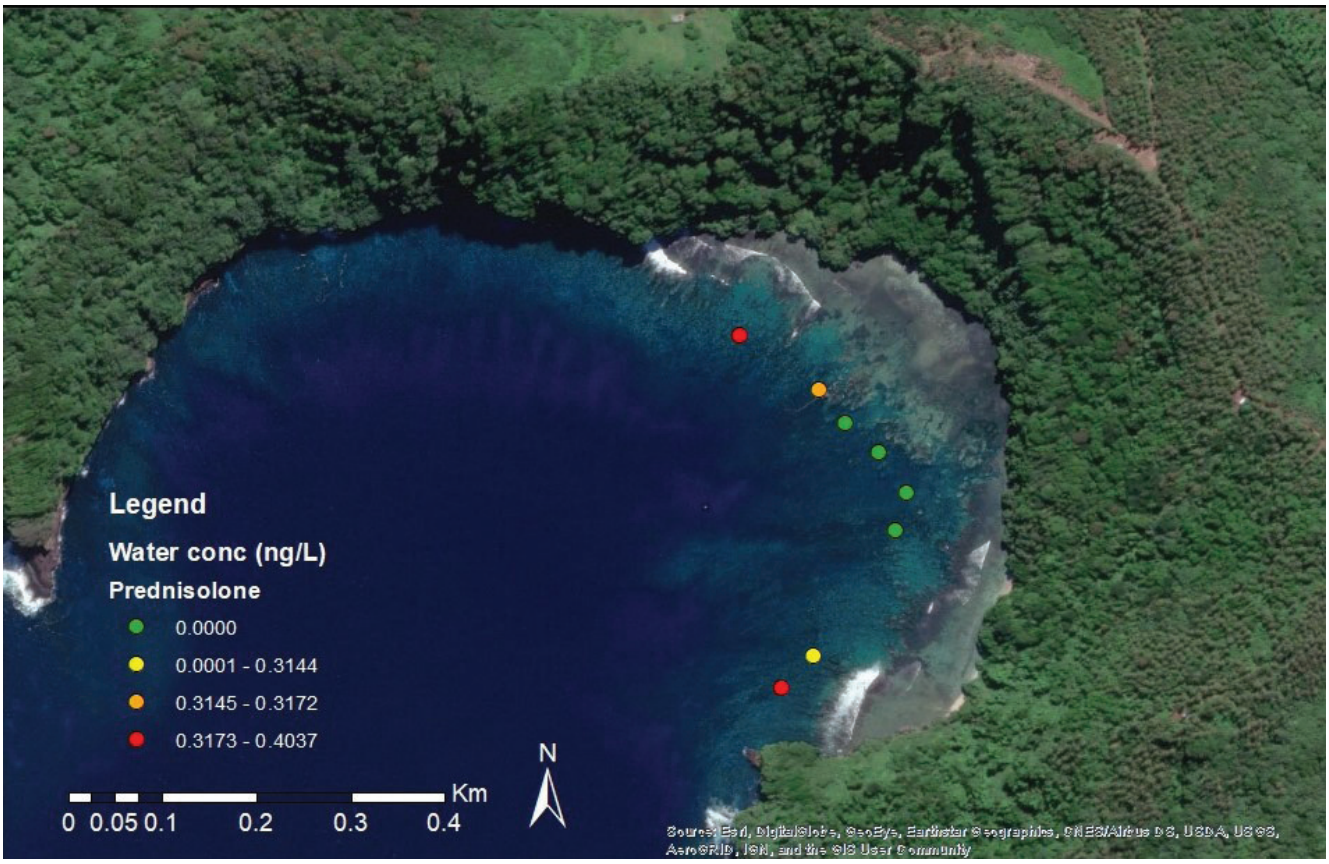

Figure 22: Water concentrations (CLAM derived) of prednisolone in Fagatele Bay.

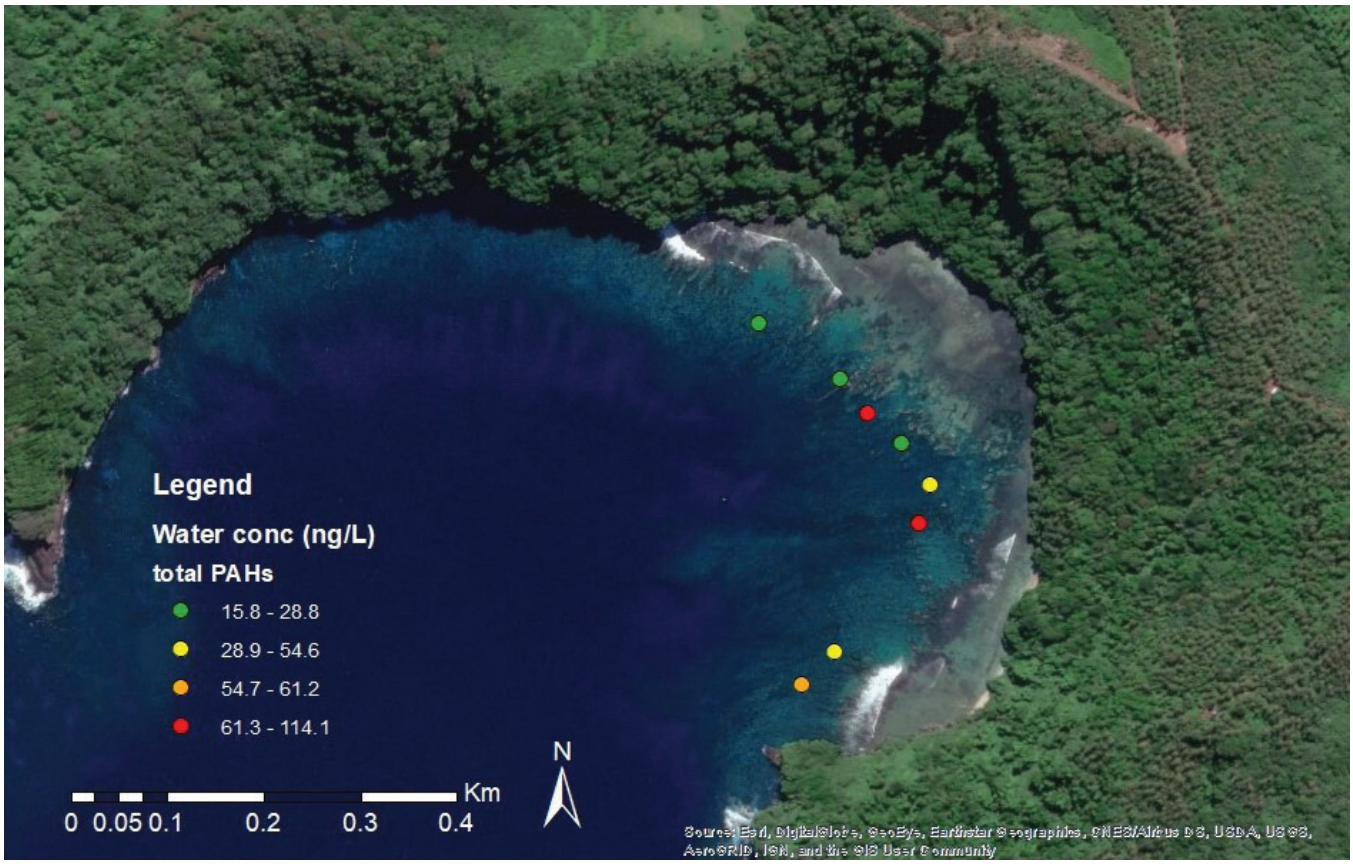

Figure 23: Water concentrations (CLAM derived) of total PAHs in Fagatele Bay.

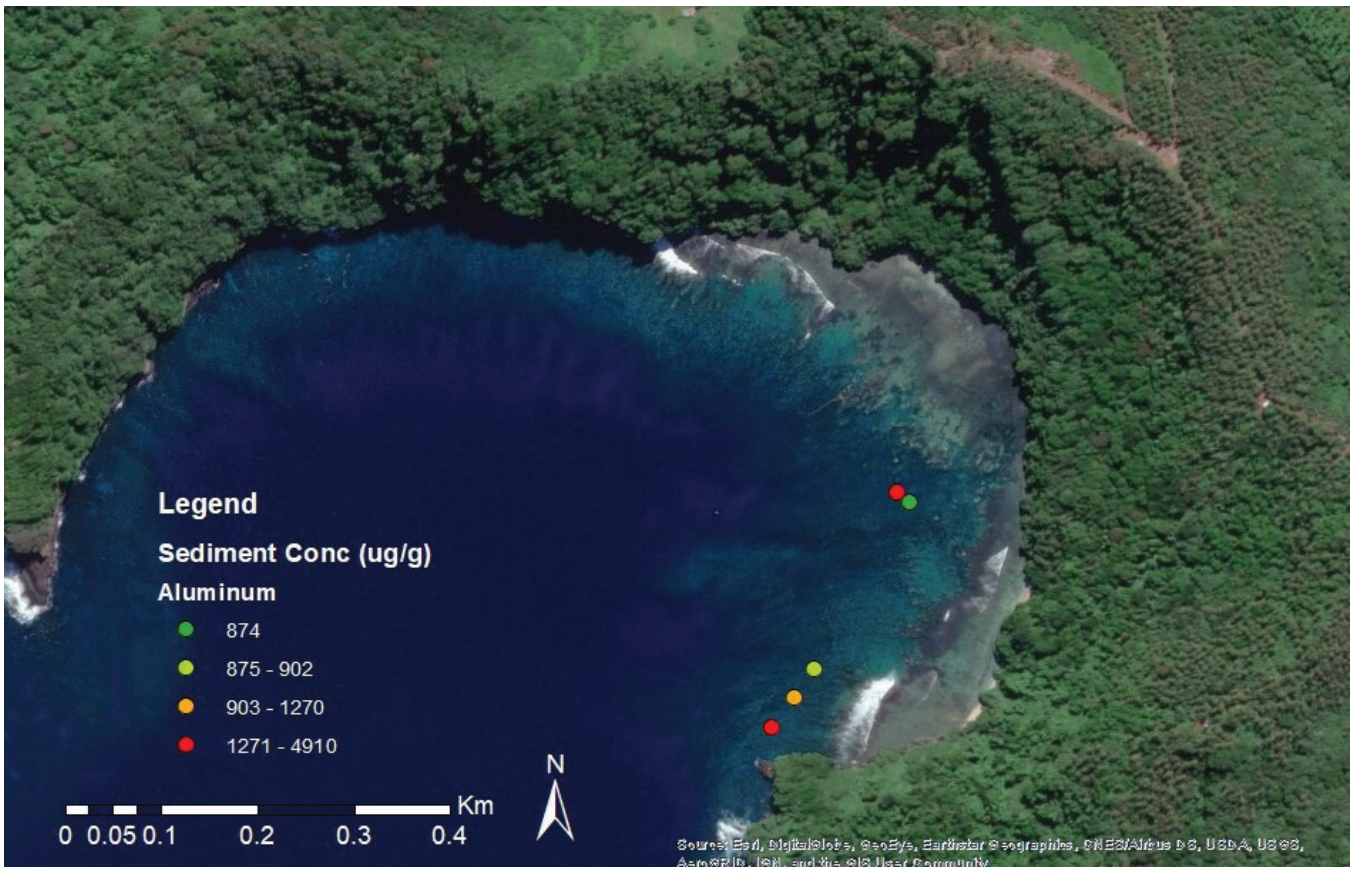

Figure 24: Concentrations of aluminum in surface sediments in Fagatele Bay.

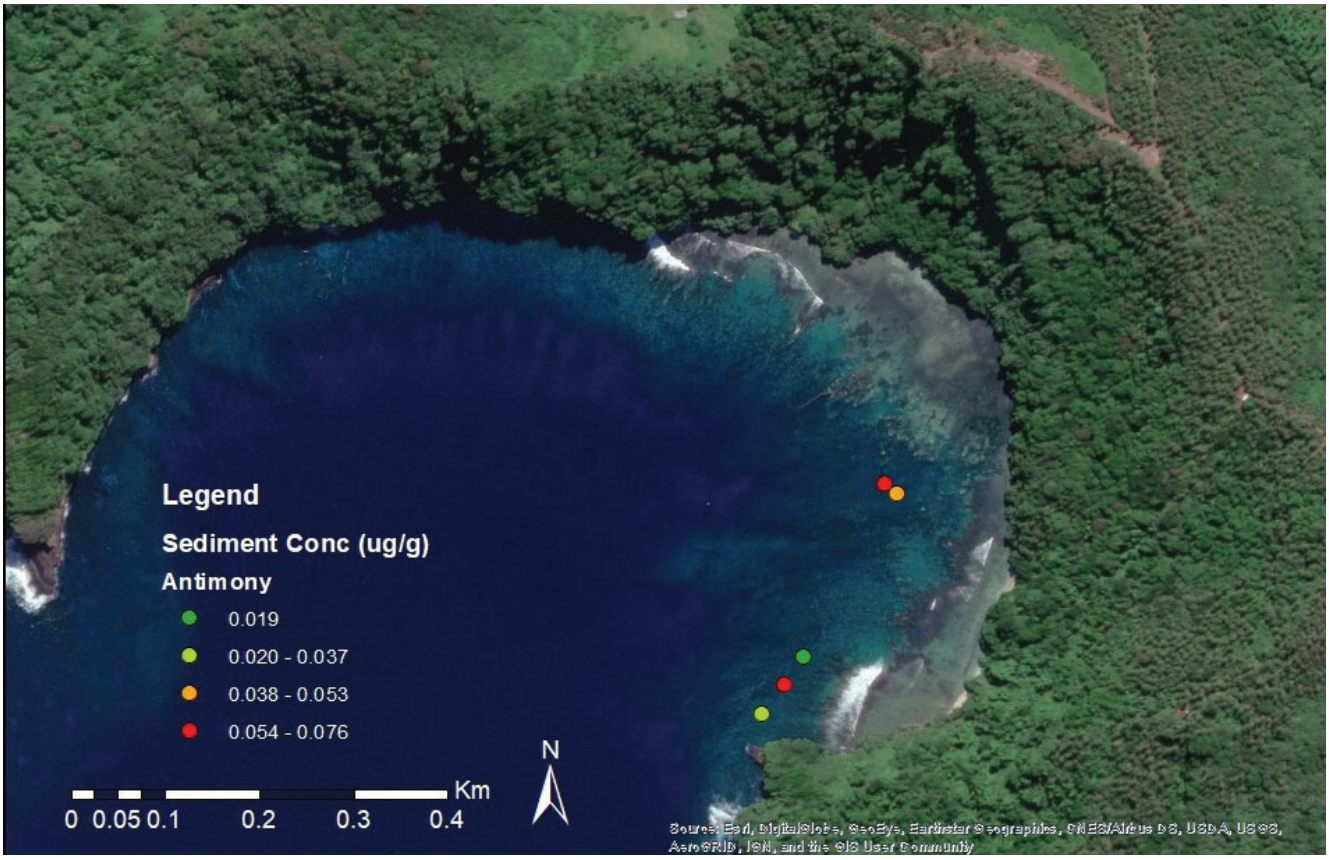

Figure 25: Concentrations of antimony in surface sediments in Fagatele Bay.

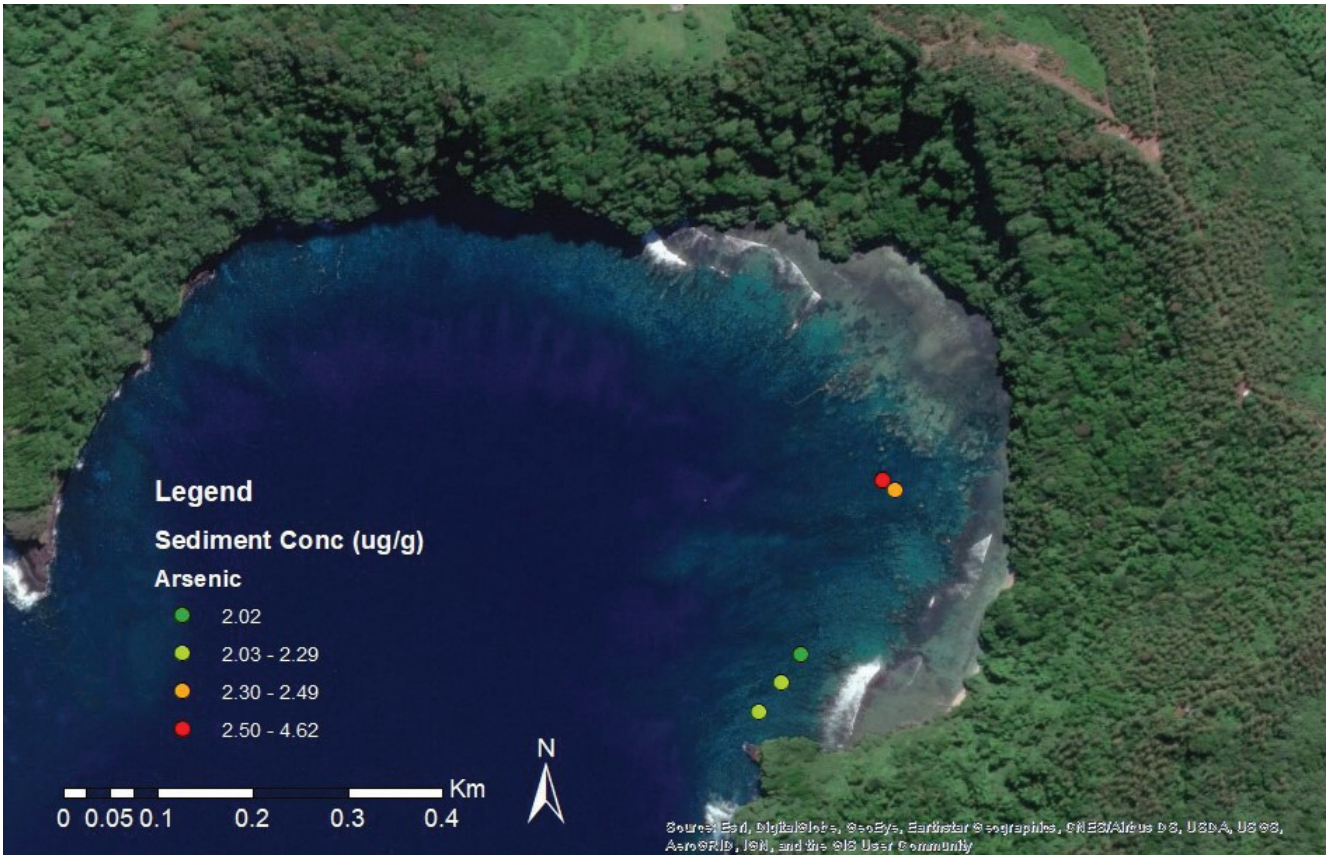

Figure 26: Concentrations of arsenic in surface sediments in Fagatele Bay.

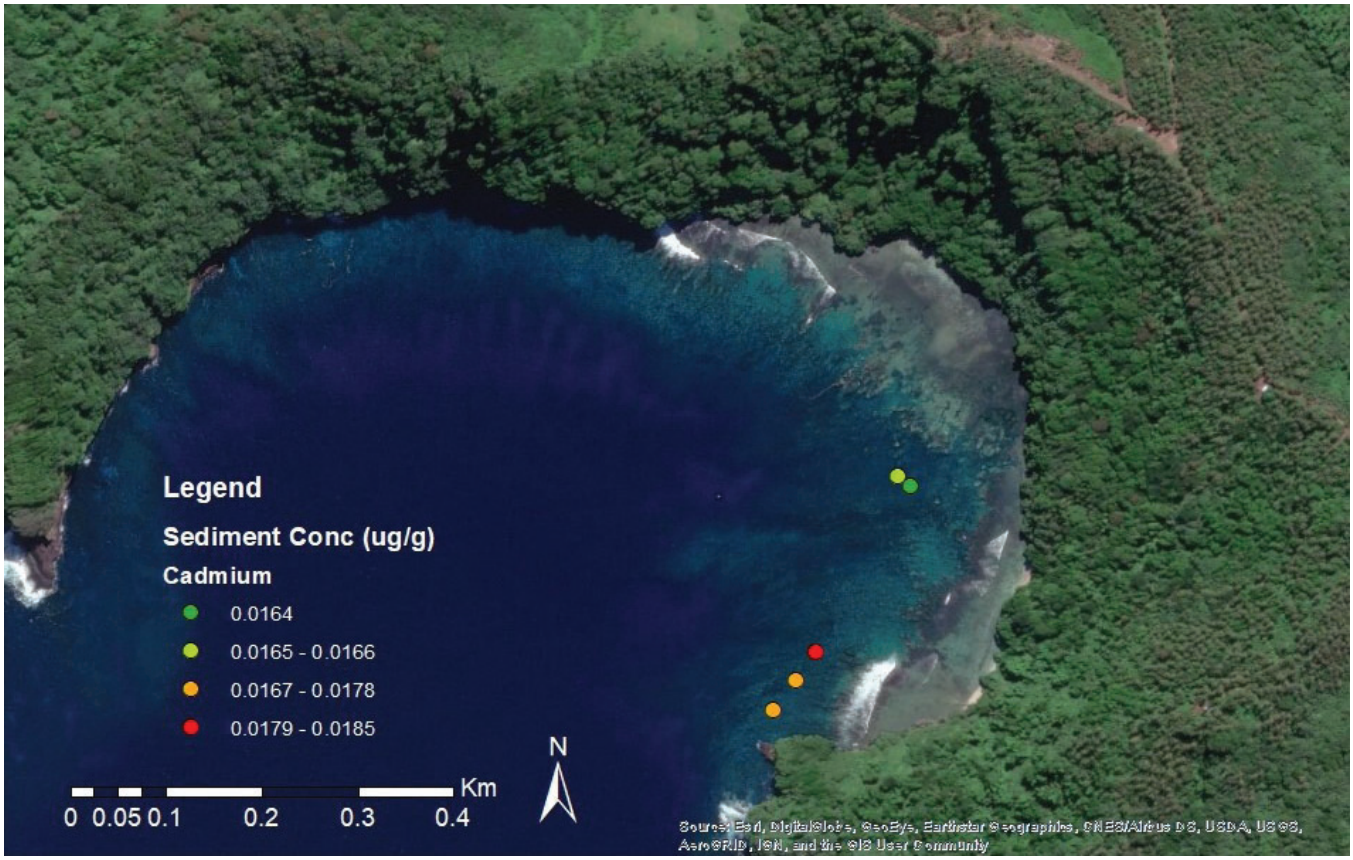

Figure 27: Concentrations of cadmium in surface sediments in Fagatele Bay.

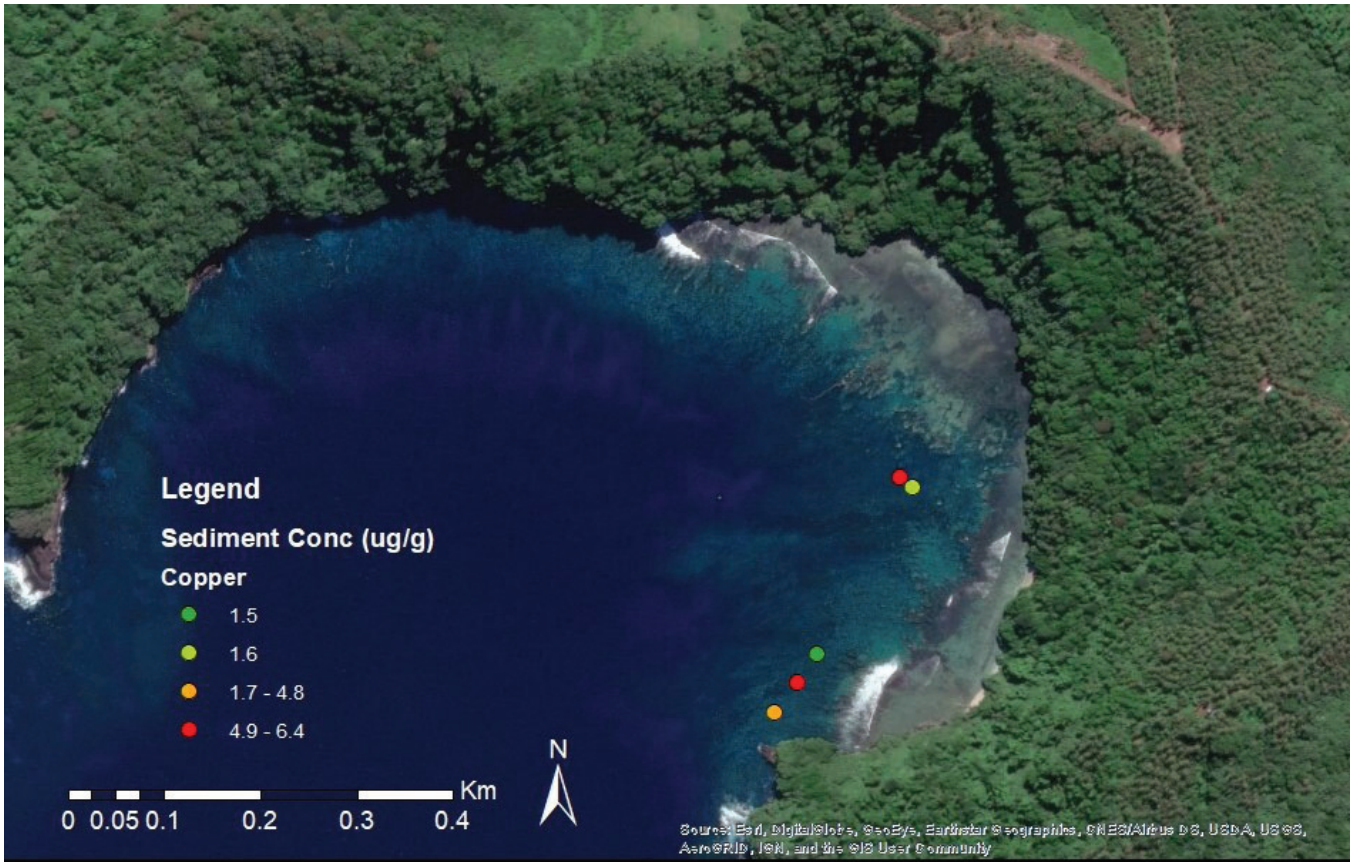

Figure 28: Concentrations of copper in surface sediments in Fagatele Bay.

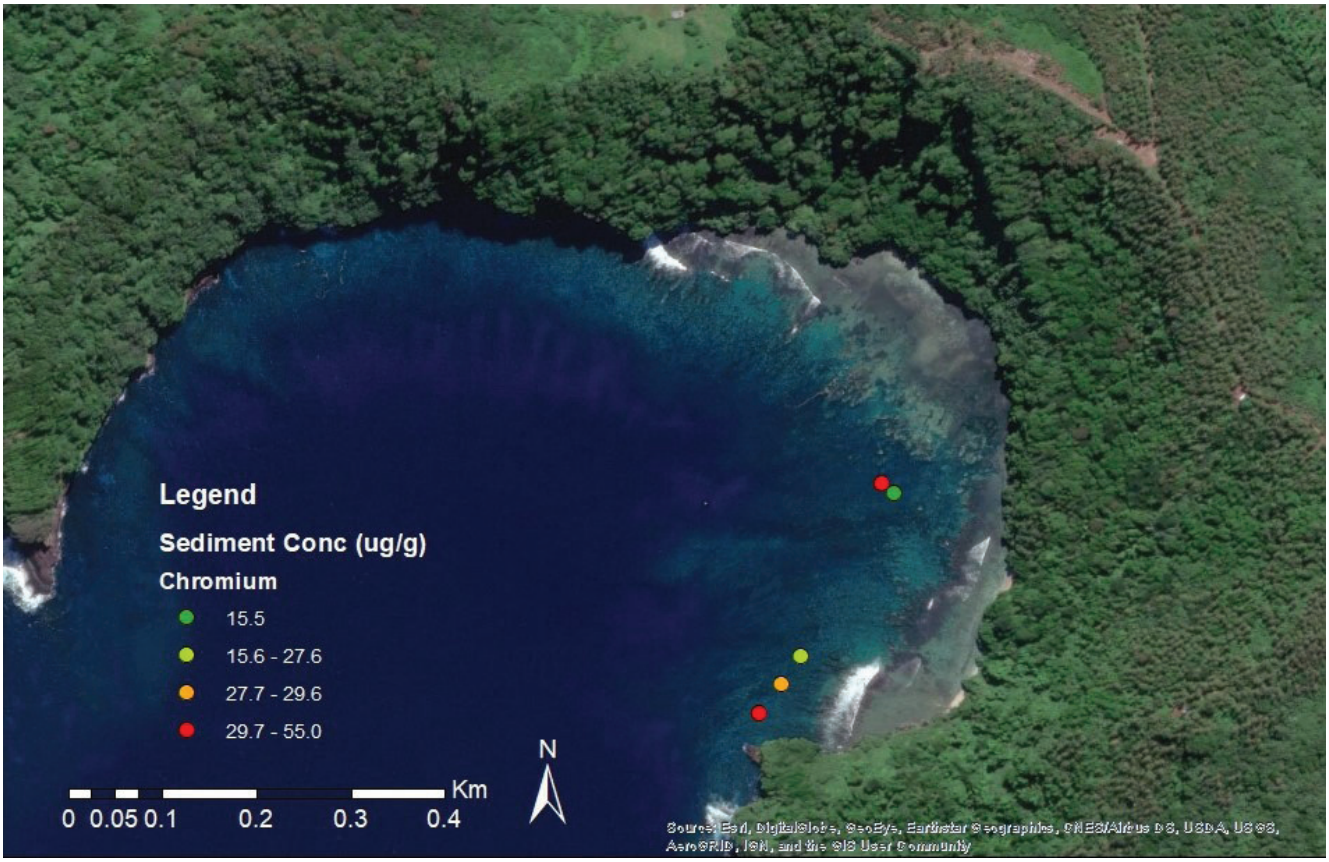

Figure 29: Concentrations of chromium in surface sediments in Fagatele Bay.

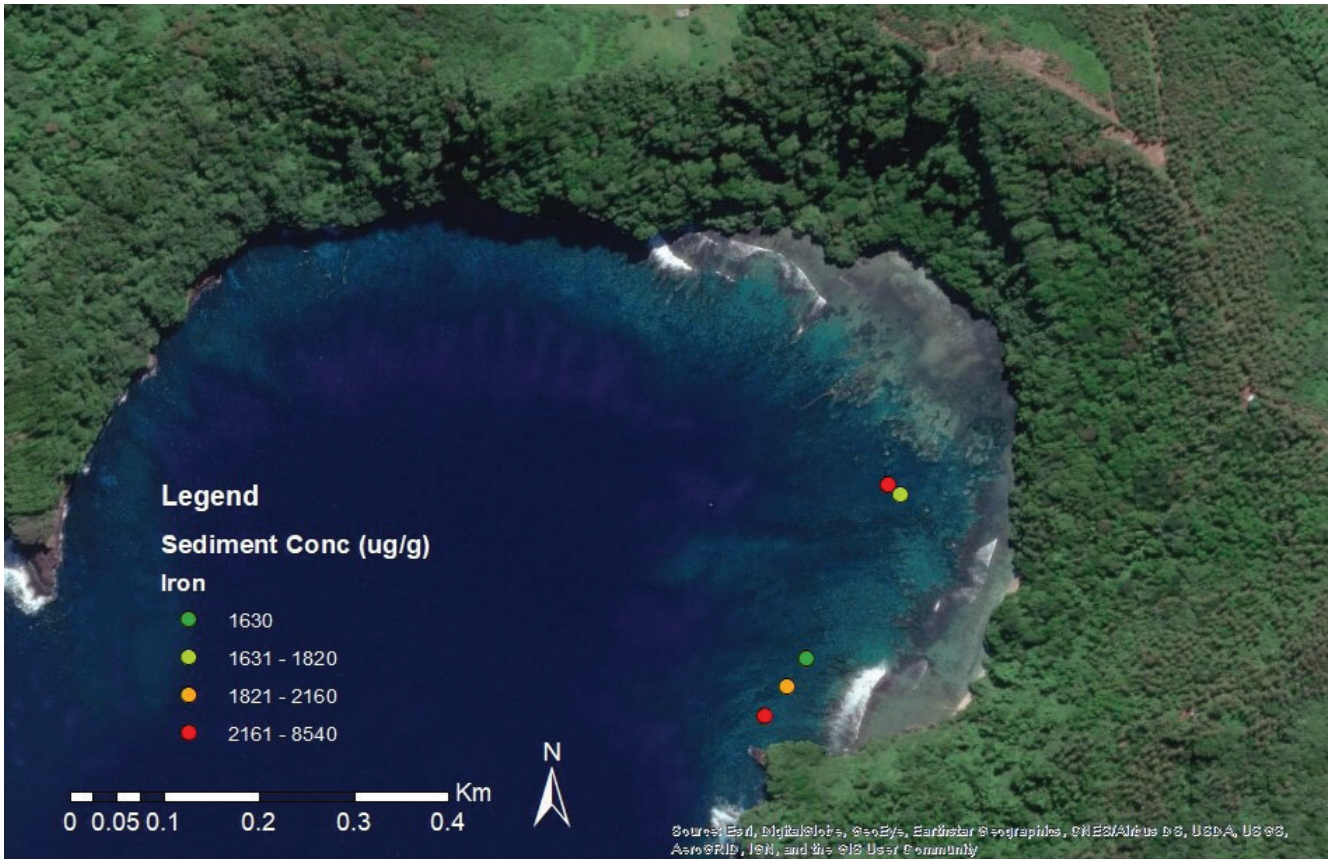

Figure 30: Concentrations of iron in surface sediments in Fagatele Bay.

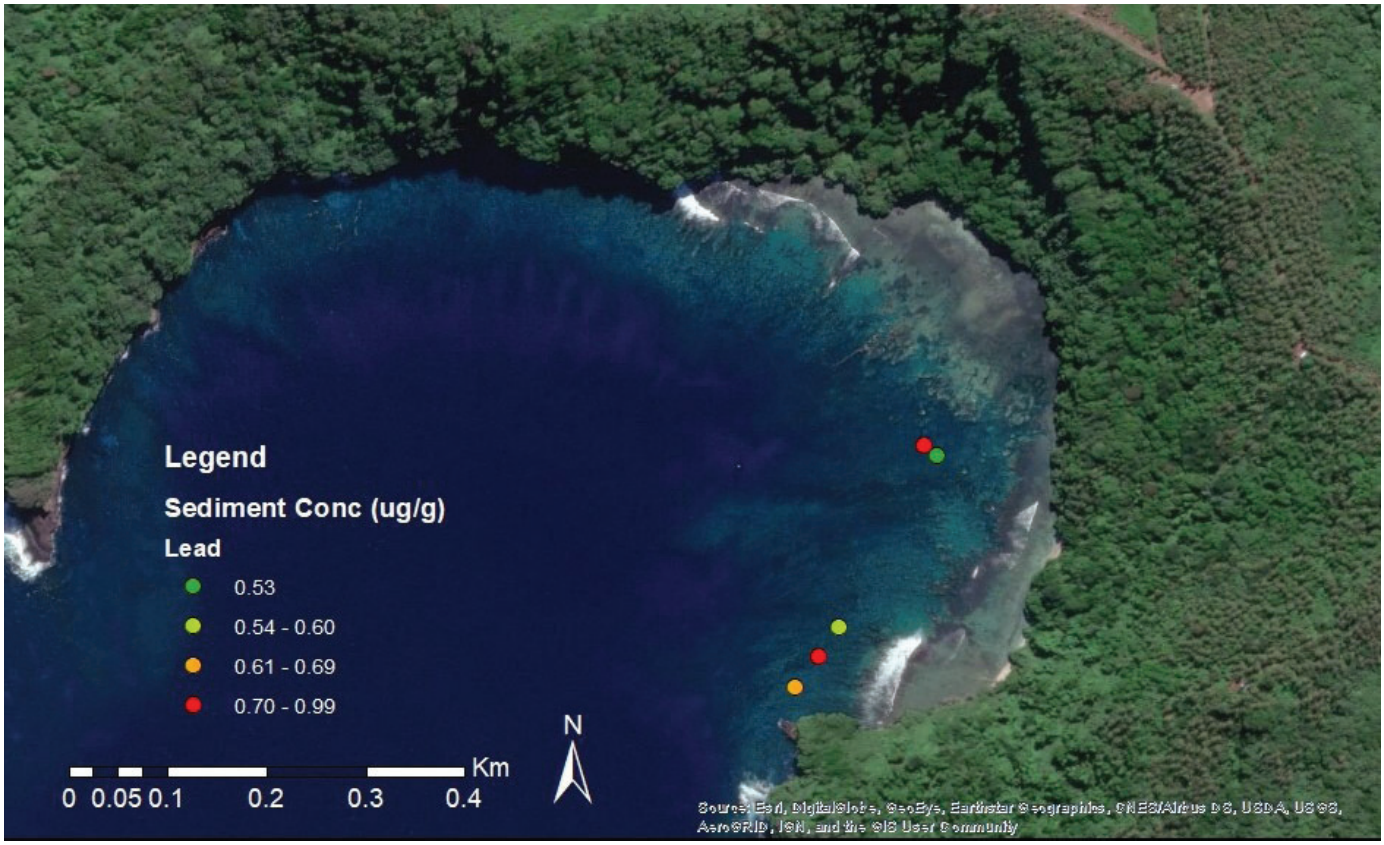

Figure 31: Concentrations of lead in surface sediments in Fagatele Bay.

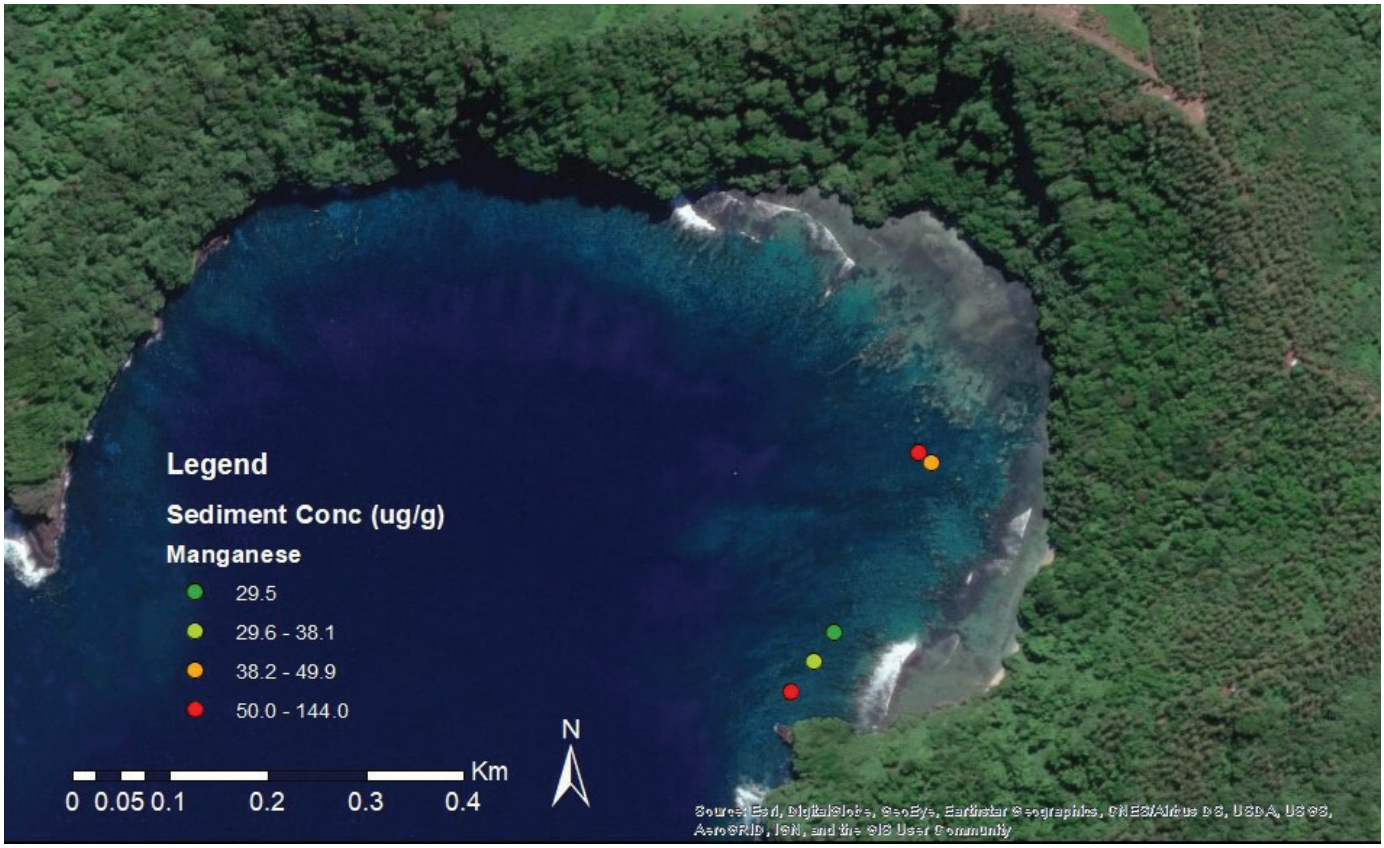

Figure 32: Concentrations of manganese in surface sediments in Fagatele Bay.

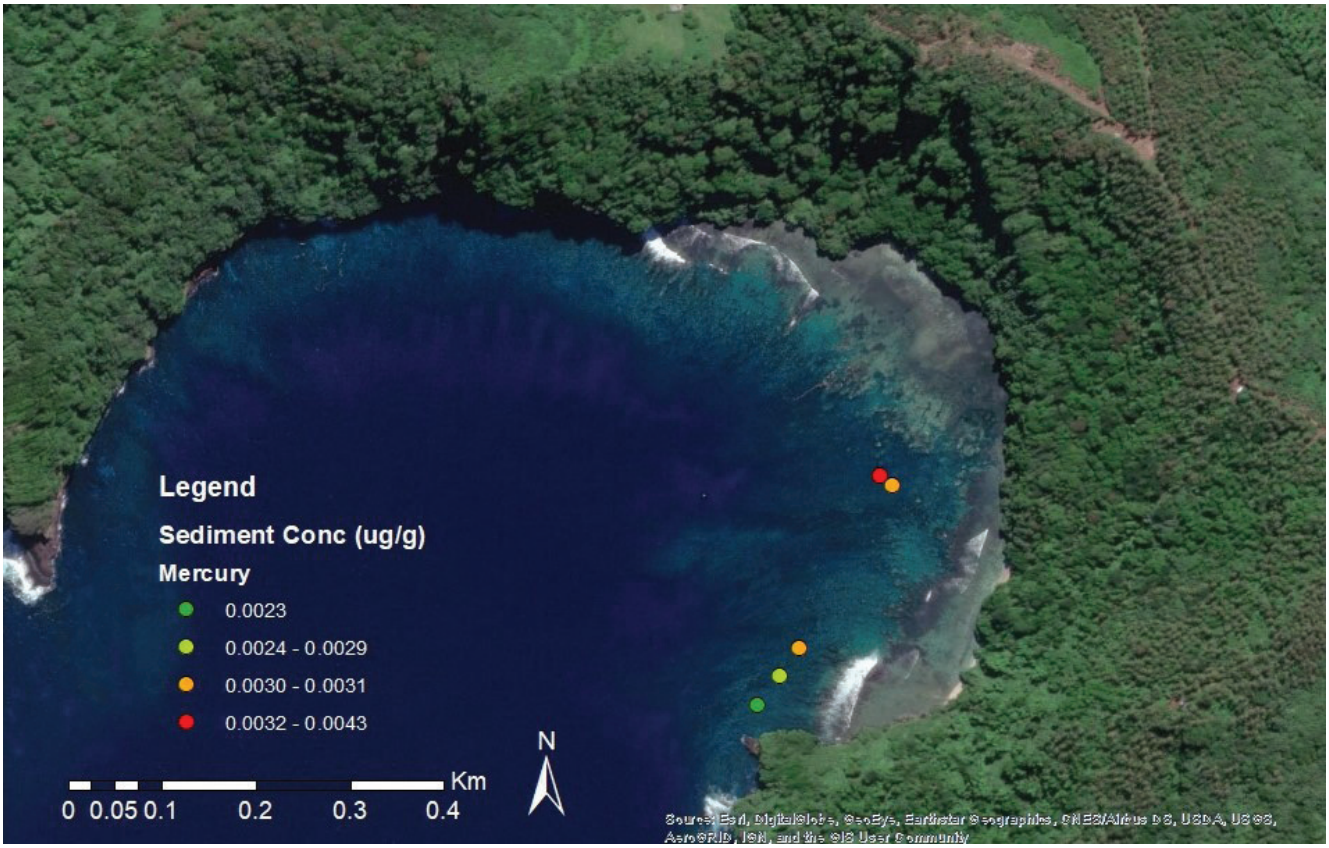

Figure 33: Concentrations of mercury in surface sediments in Fagatele Bay.

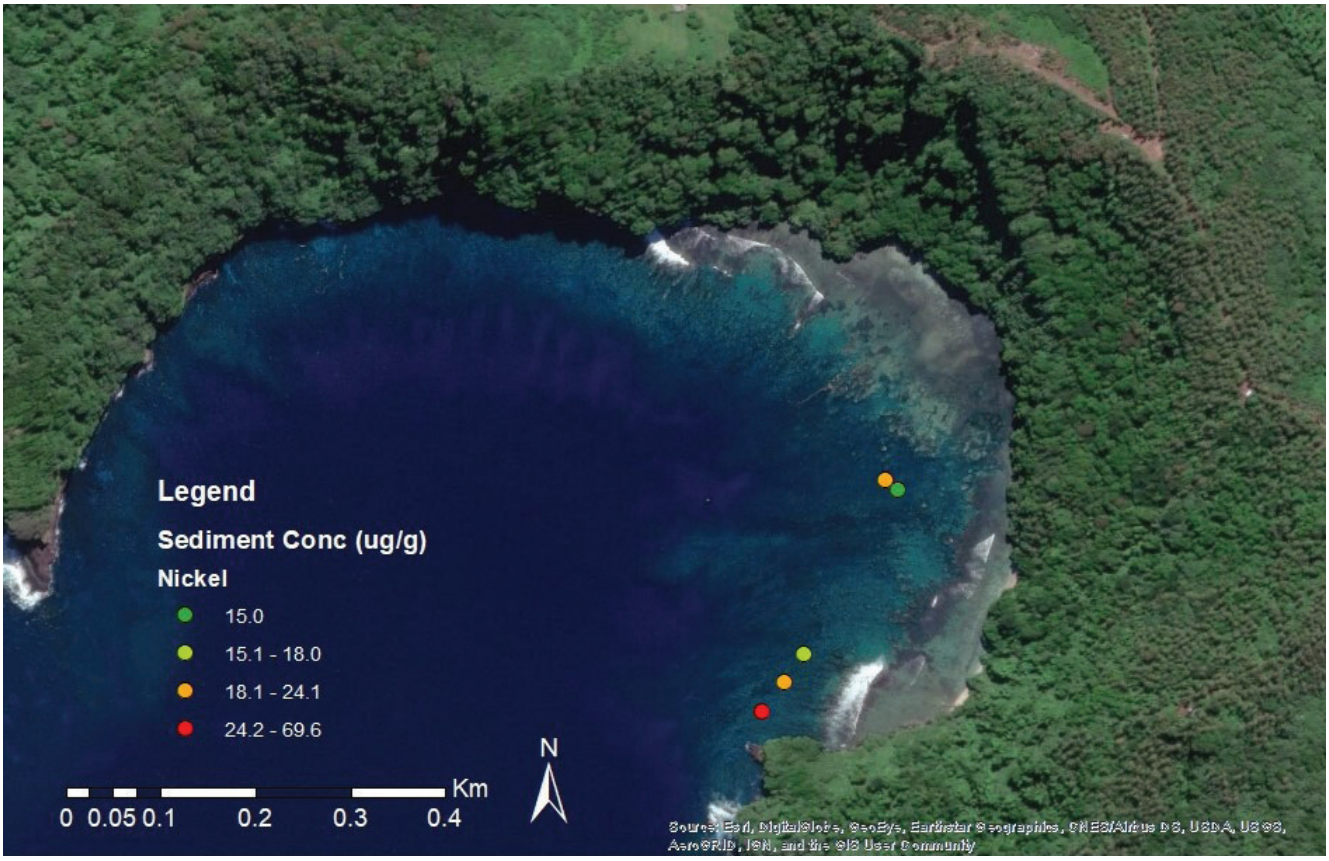

Figure 34: Concentrations of nickel in surface sediments in Fagatele Bay.

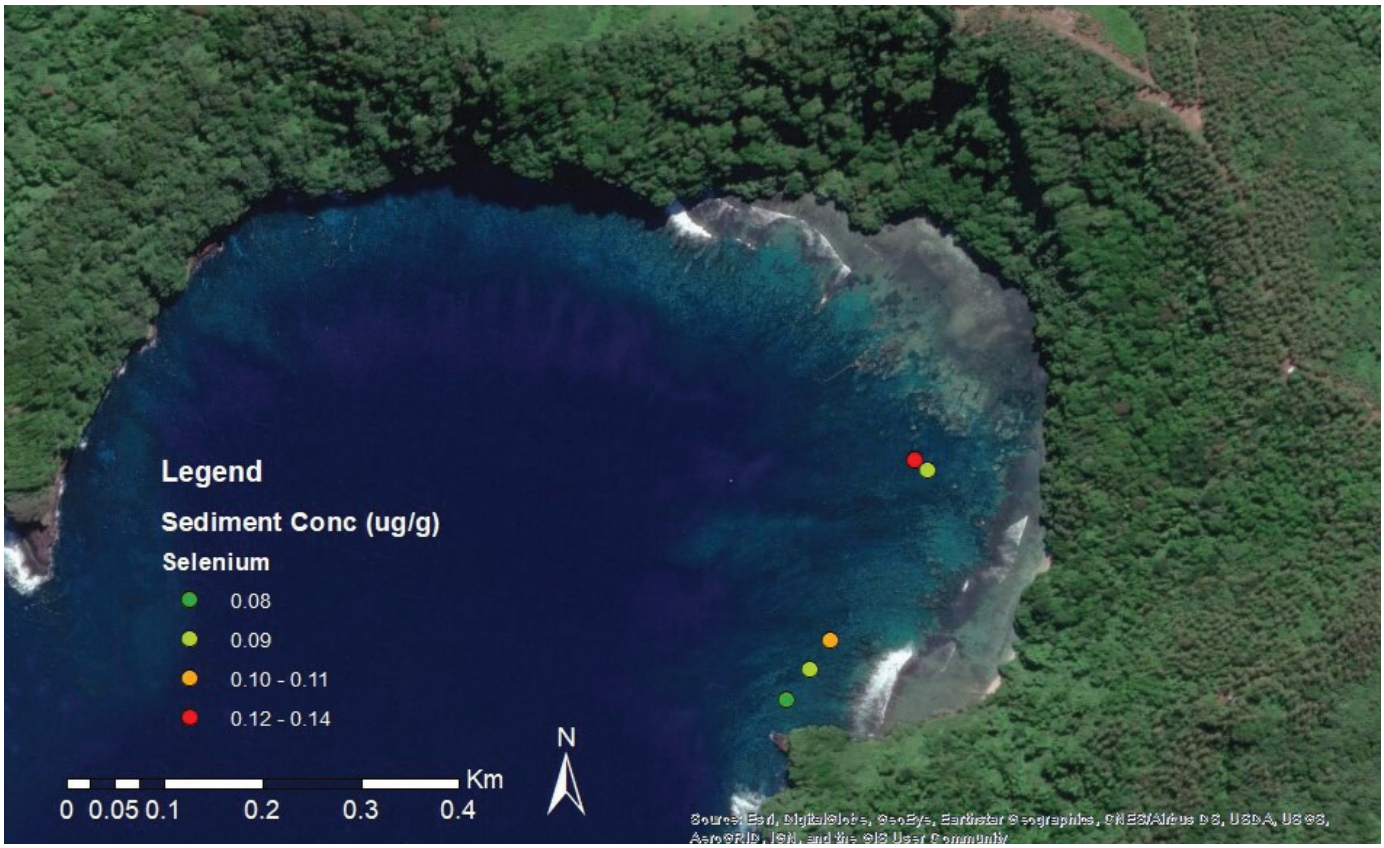

Figure 35: Concentrations of selenium in surface sediments in Fagatele Bay.

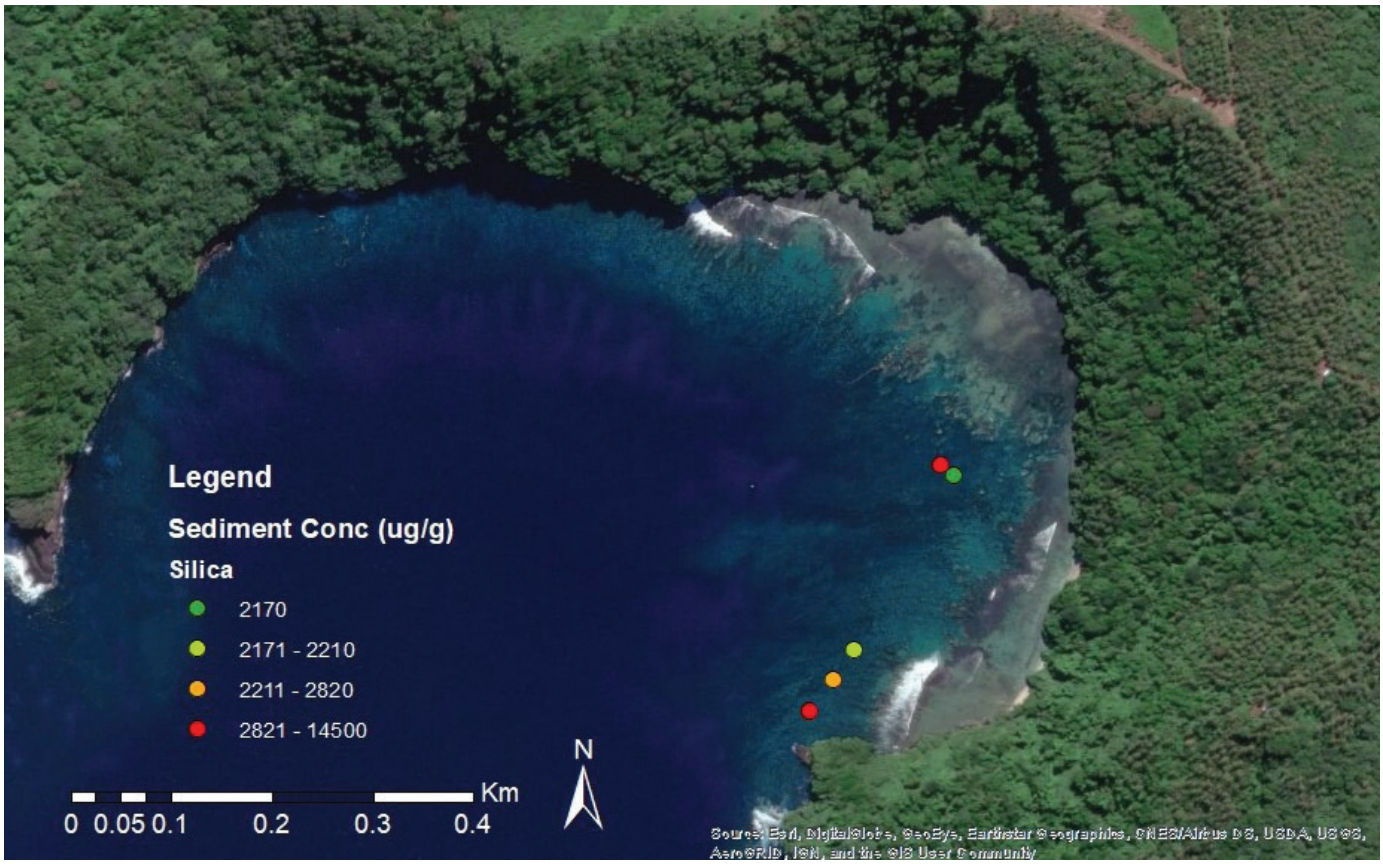

Figure 36: Concentrations of silica in surface sediments in Fagatele Bay.

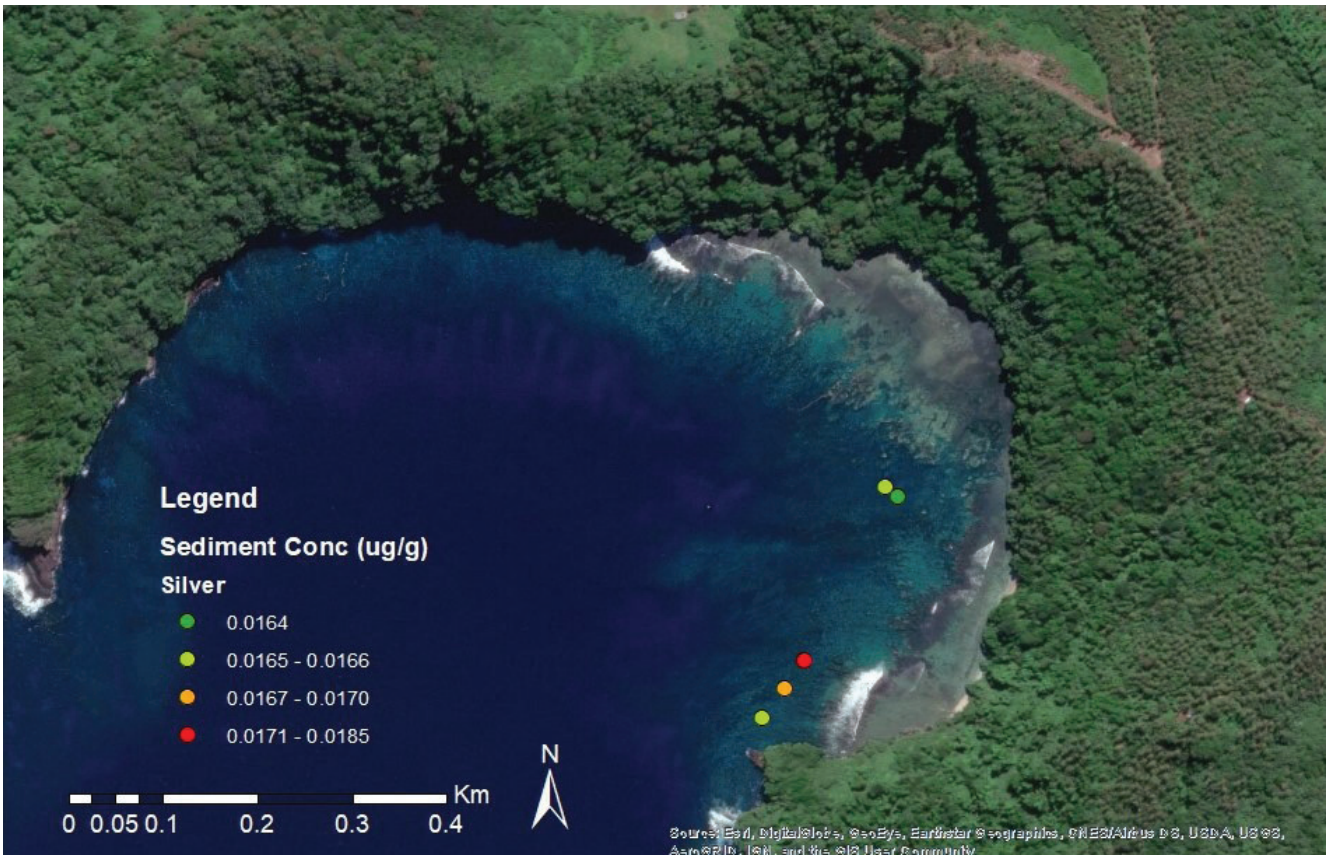

Figure 37: Concentrations of silver in surface sediments in Fagatele Bay.

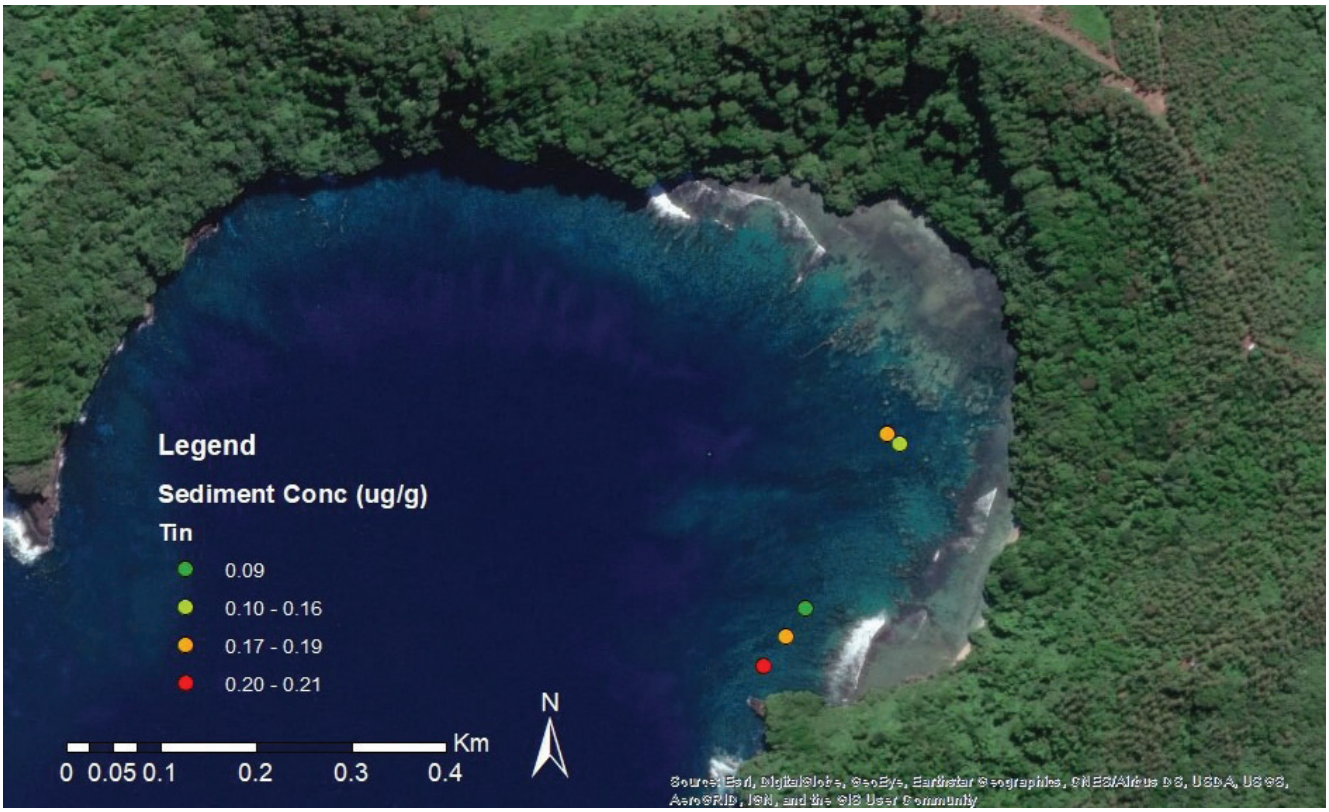

Figure 38: Concentrations of tin in surface sediments in Fagatele Bay.

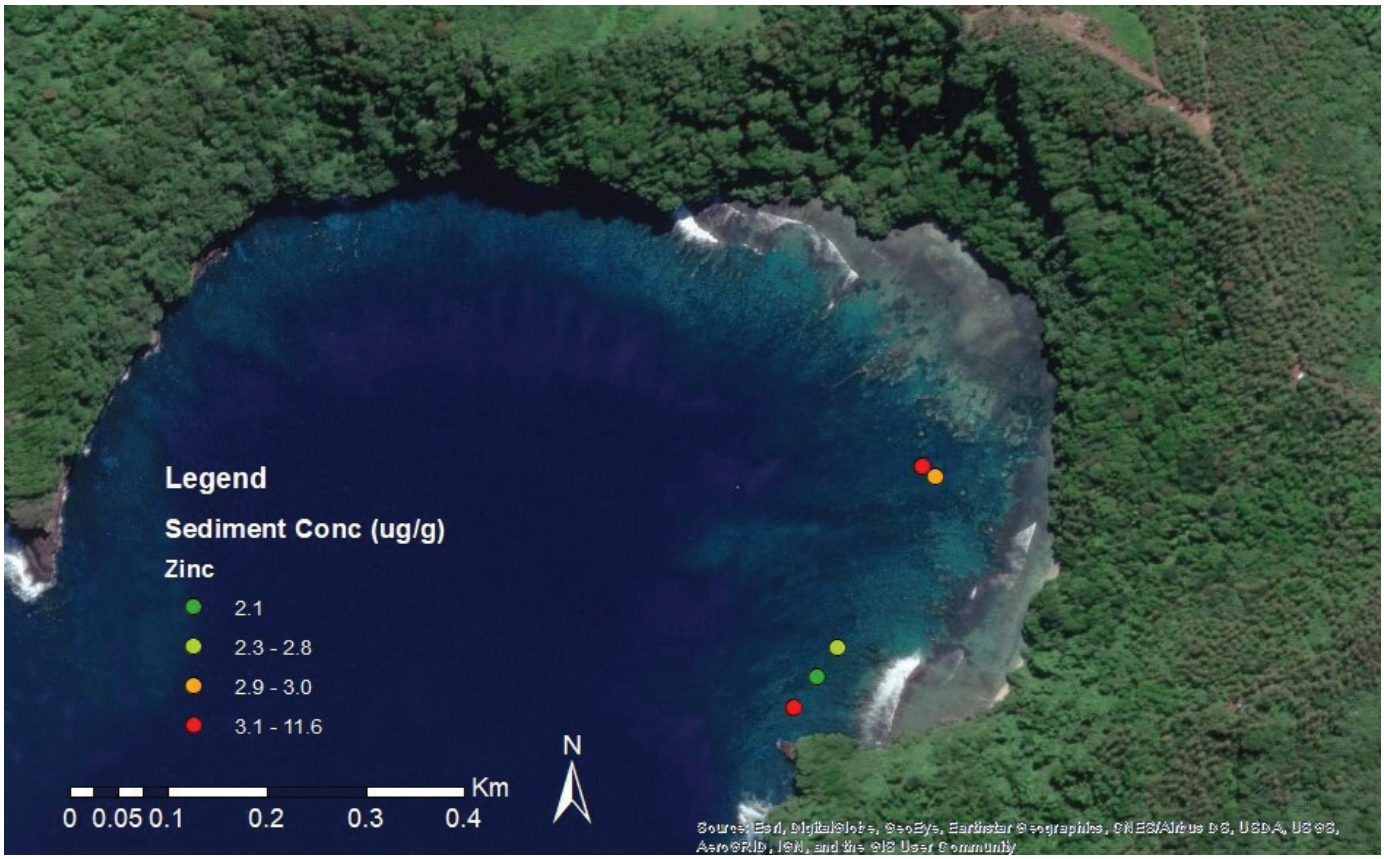

Figure 39: Concentrations of zinc in surface sediments in Fagatele Bay.

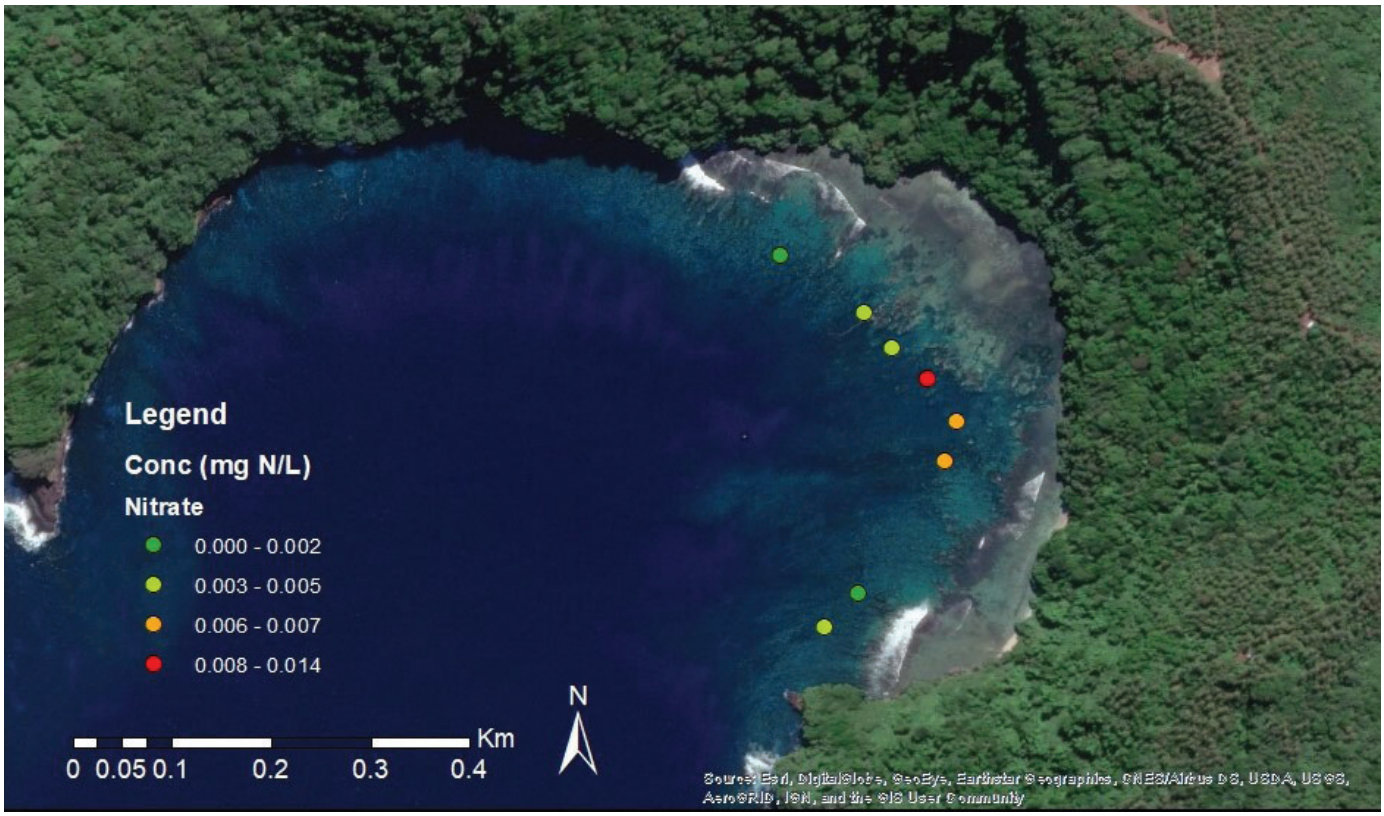

Figure 40: Water column (bottom water) concentrations of nitrate in Fagatele Bay.

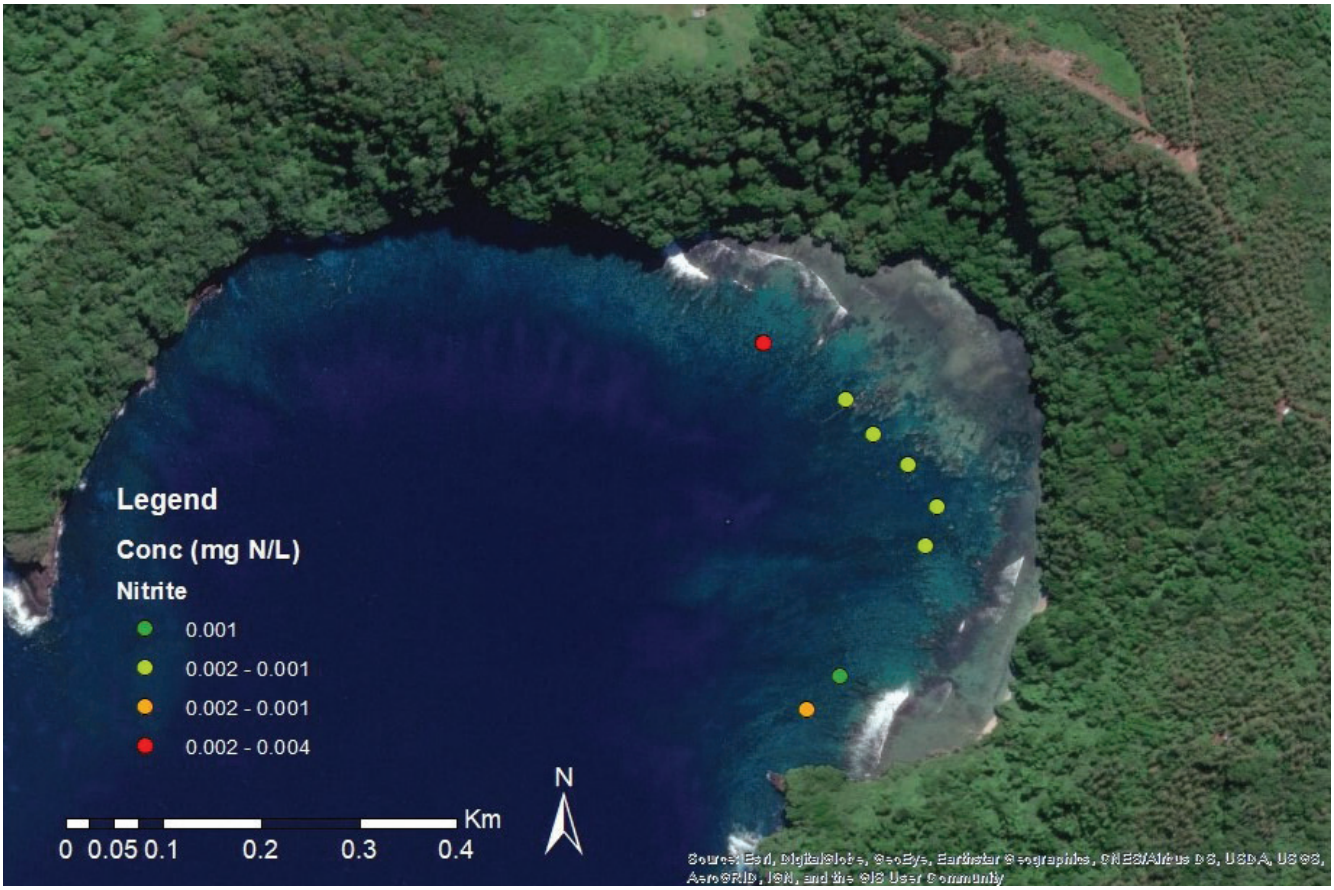

Figure 41: Water column (bottom water) concentrations of nitrite in Fagatele Bay.

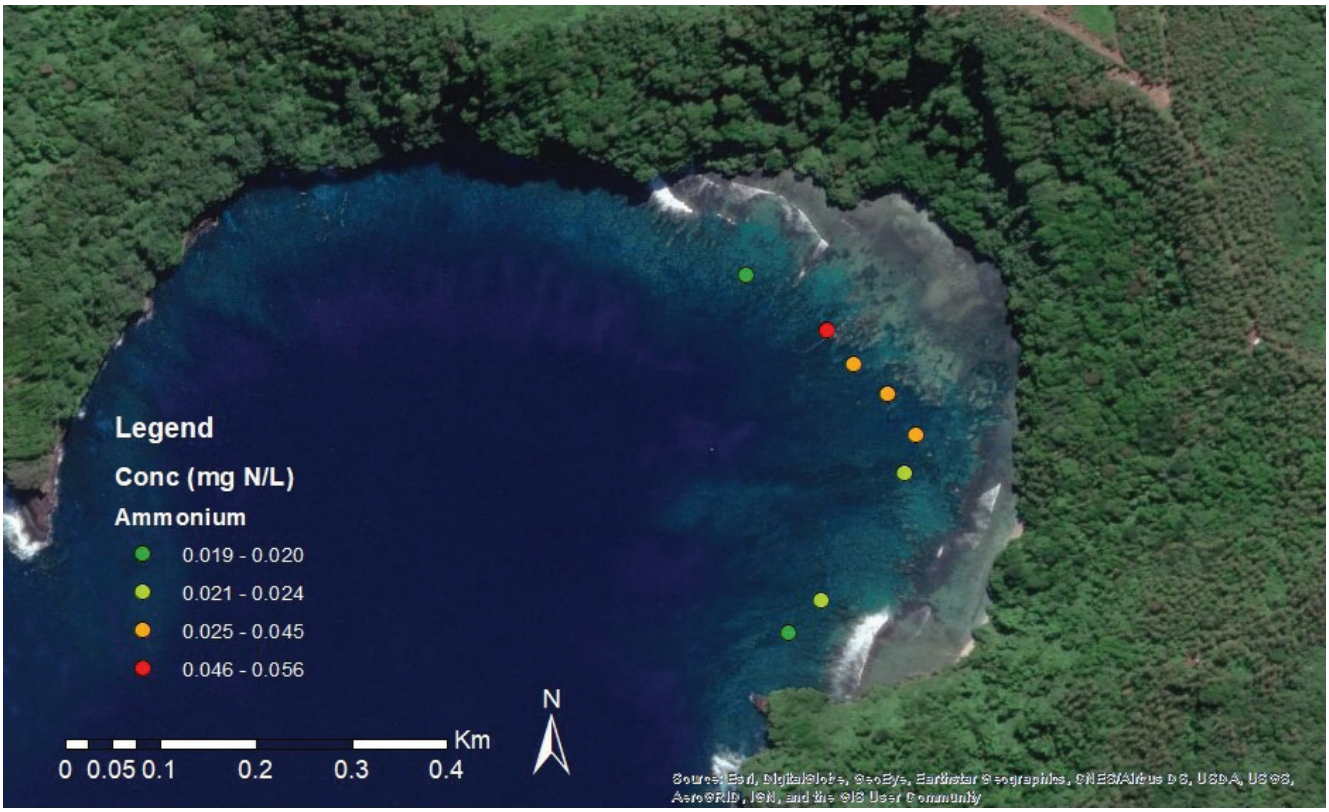

Figure 42: Water column (bottom water) concentrations of ammonium in Fagatele Bay.

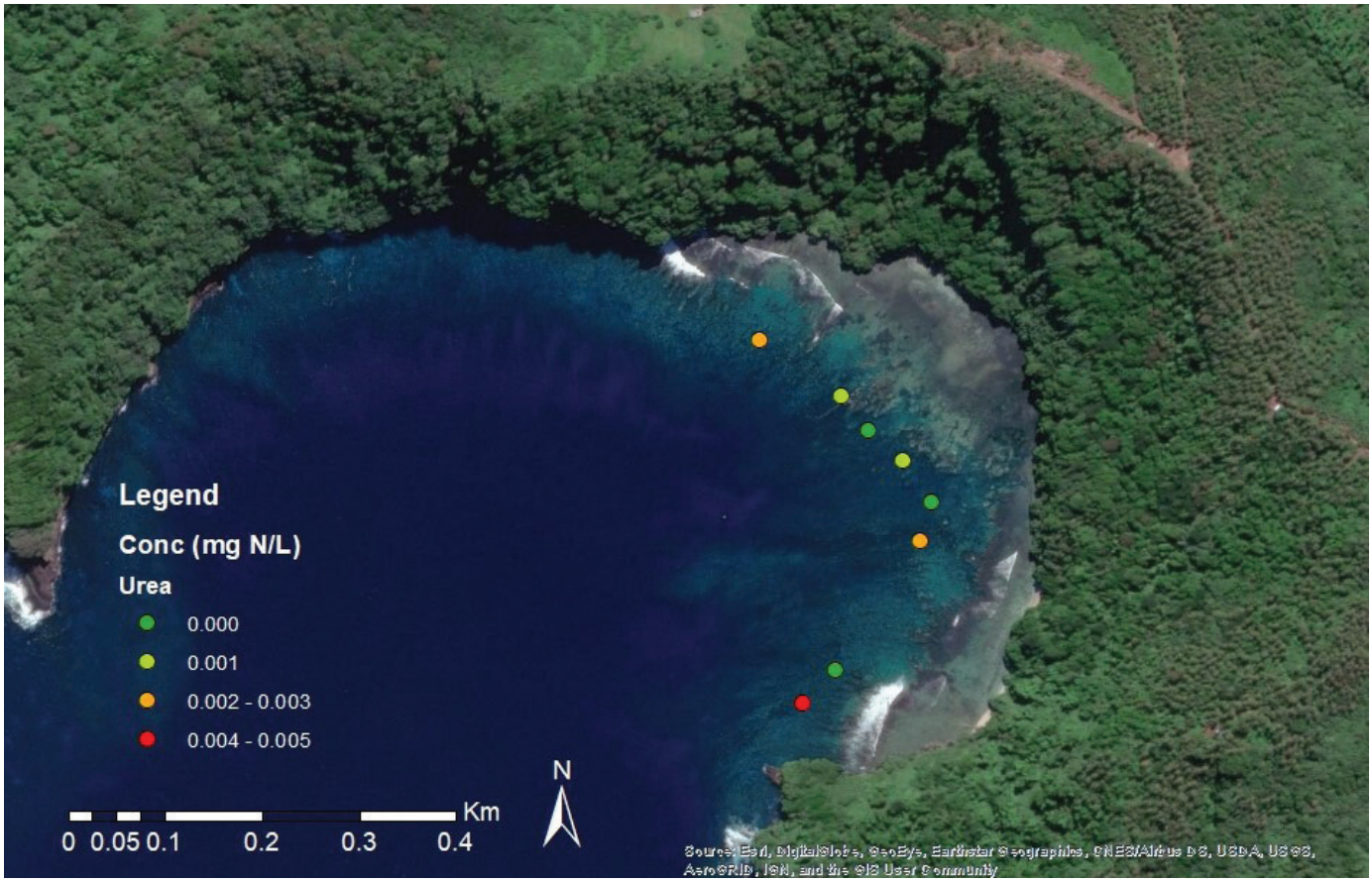

Figure 43: Water column (bottom water) concentrations of urea in Fagatele Bay.

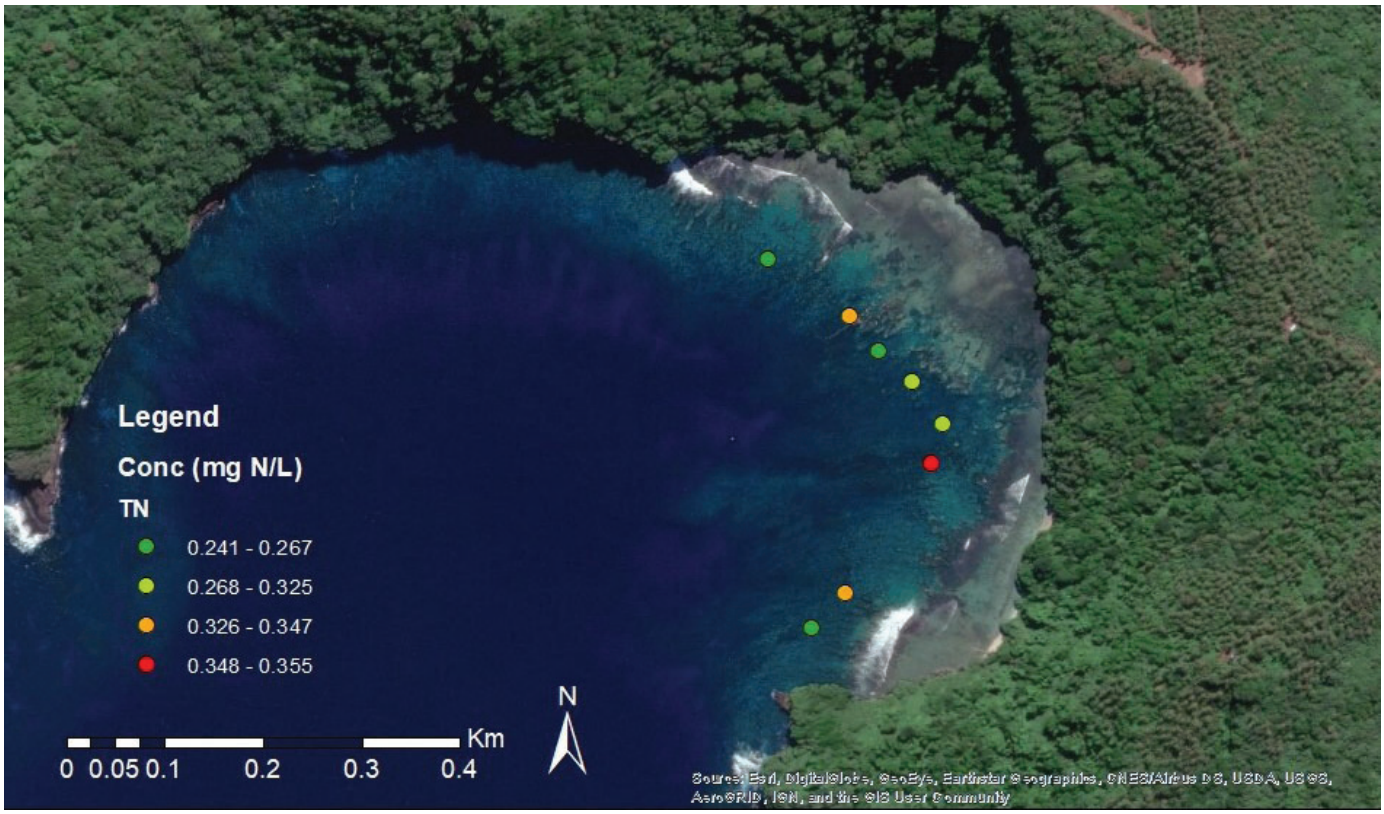

Figure 44: Water column (bottom water) concentrations of total nitrogen in Fagatele Bay.

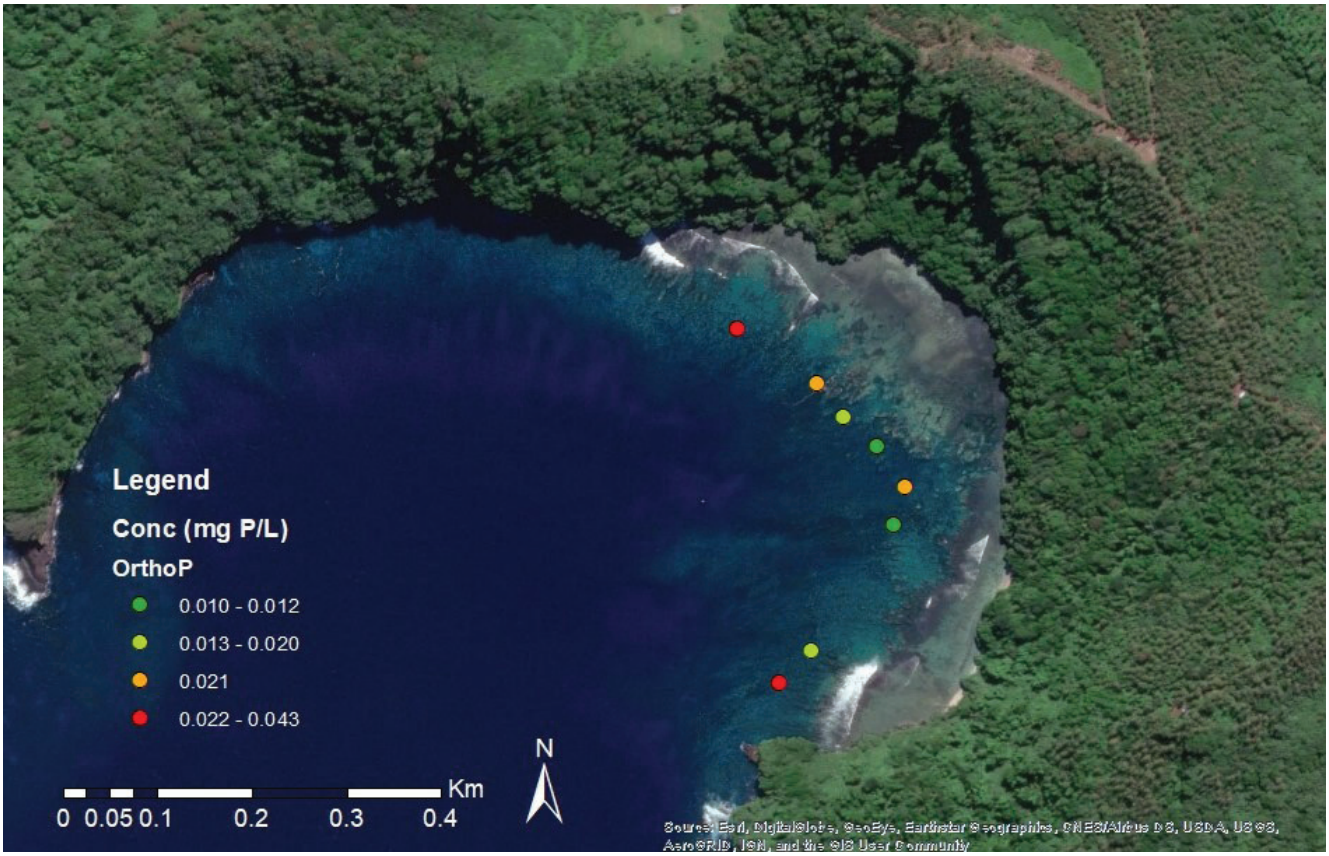

Figure 45: Water column (bottom water) concentrations of orthophosphate in Fagatele Bay.

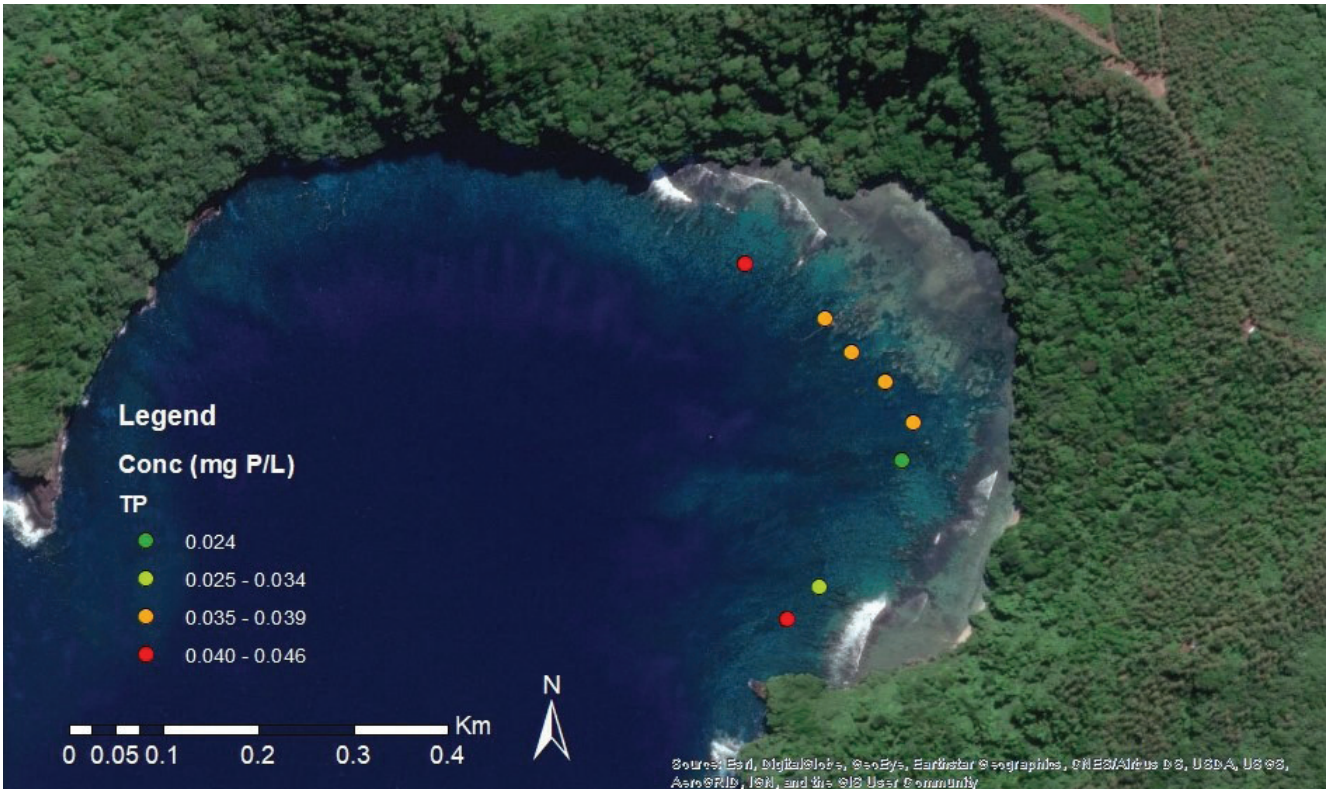

Figure 46: Water column (bottom water) concentrations of total phosphorus in Fagatele Bay.

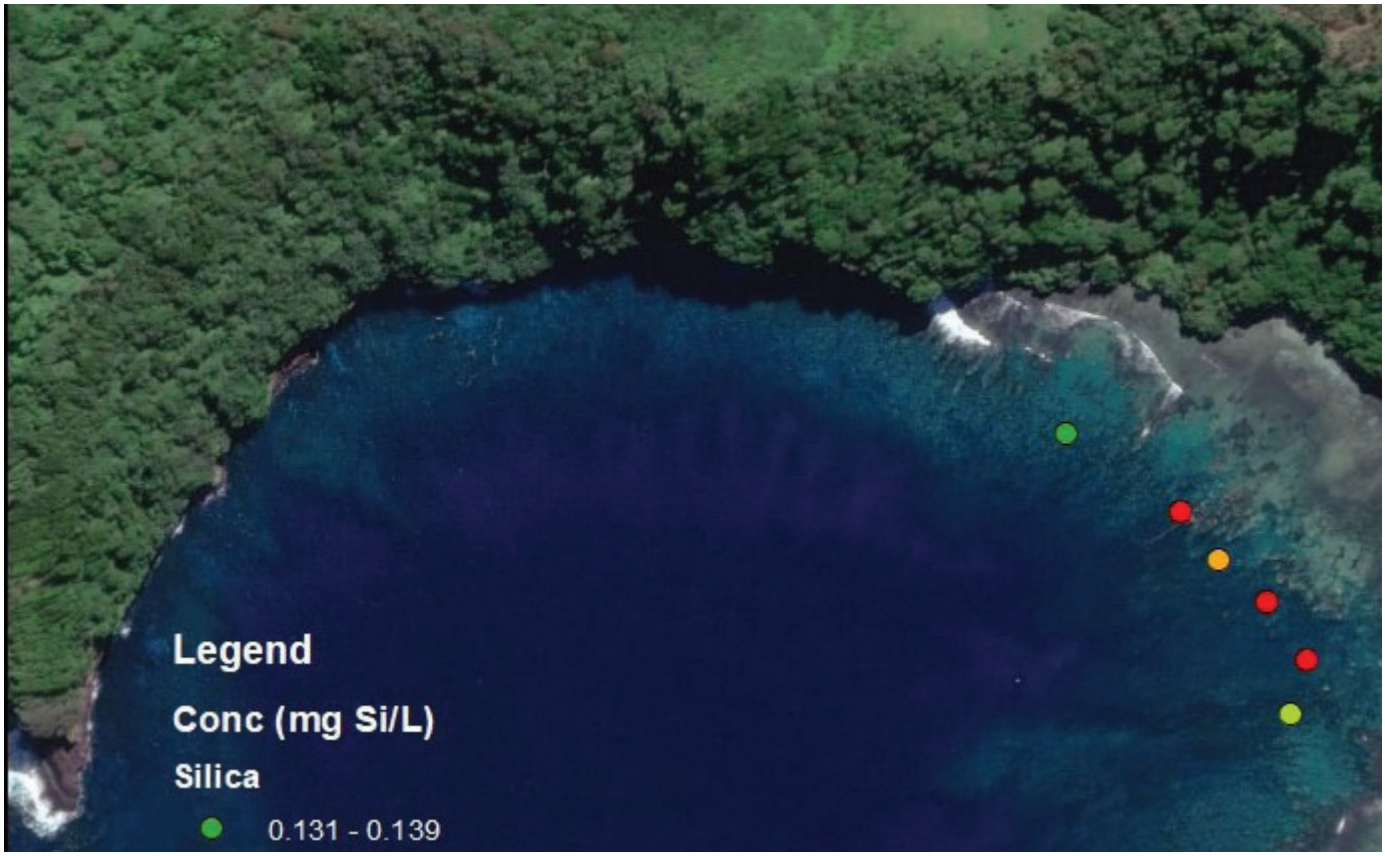

Figure 47: Water column (bottom water) concentrations of silica in Fagatele Bay.

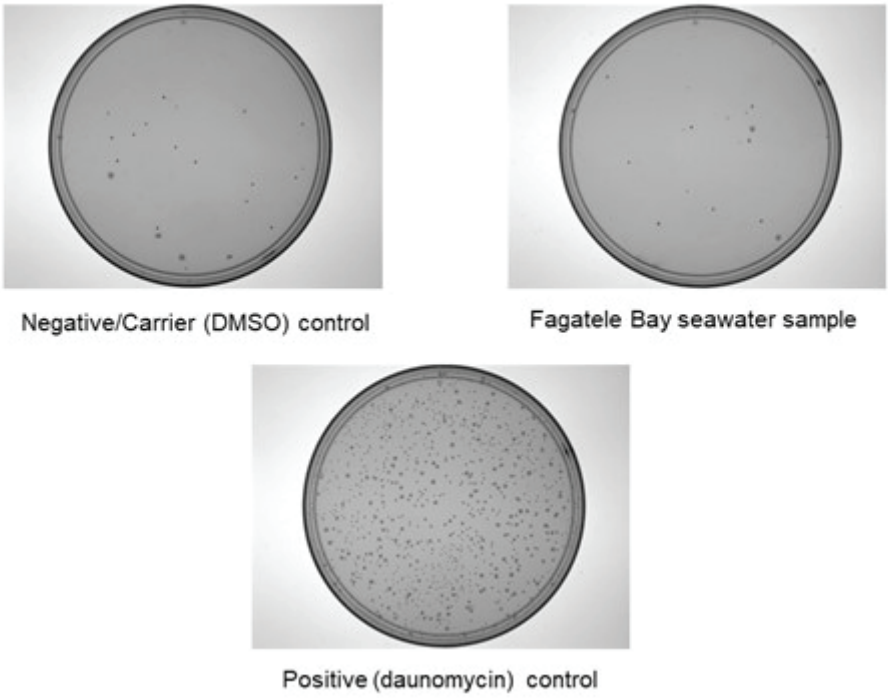

Figure 48: Plates from Ames test.

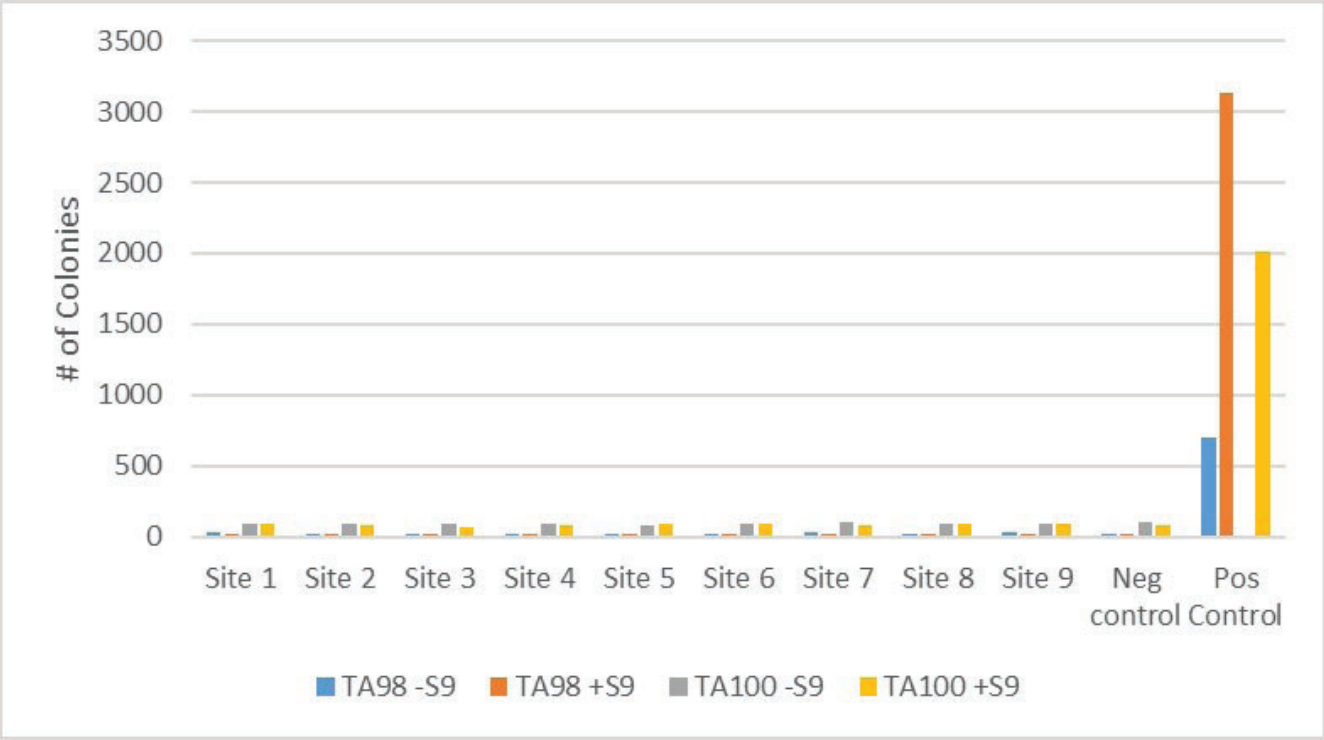

Figure 49: Ames test results (colony enumeration) for Fagatele Bay NMS samples.

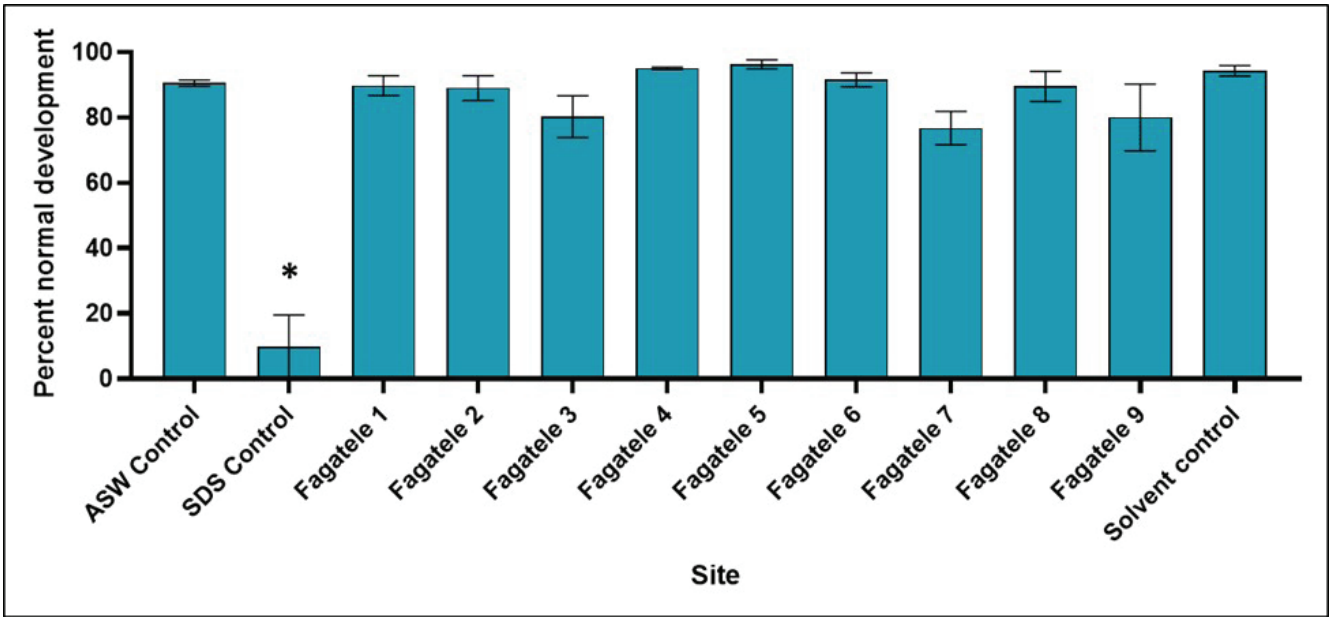

Figure 50: Results of sea urchin embryo development toxicity test with *Lytechinus variegatus*.

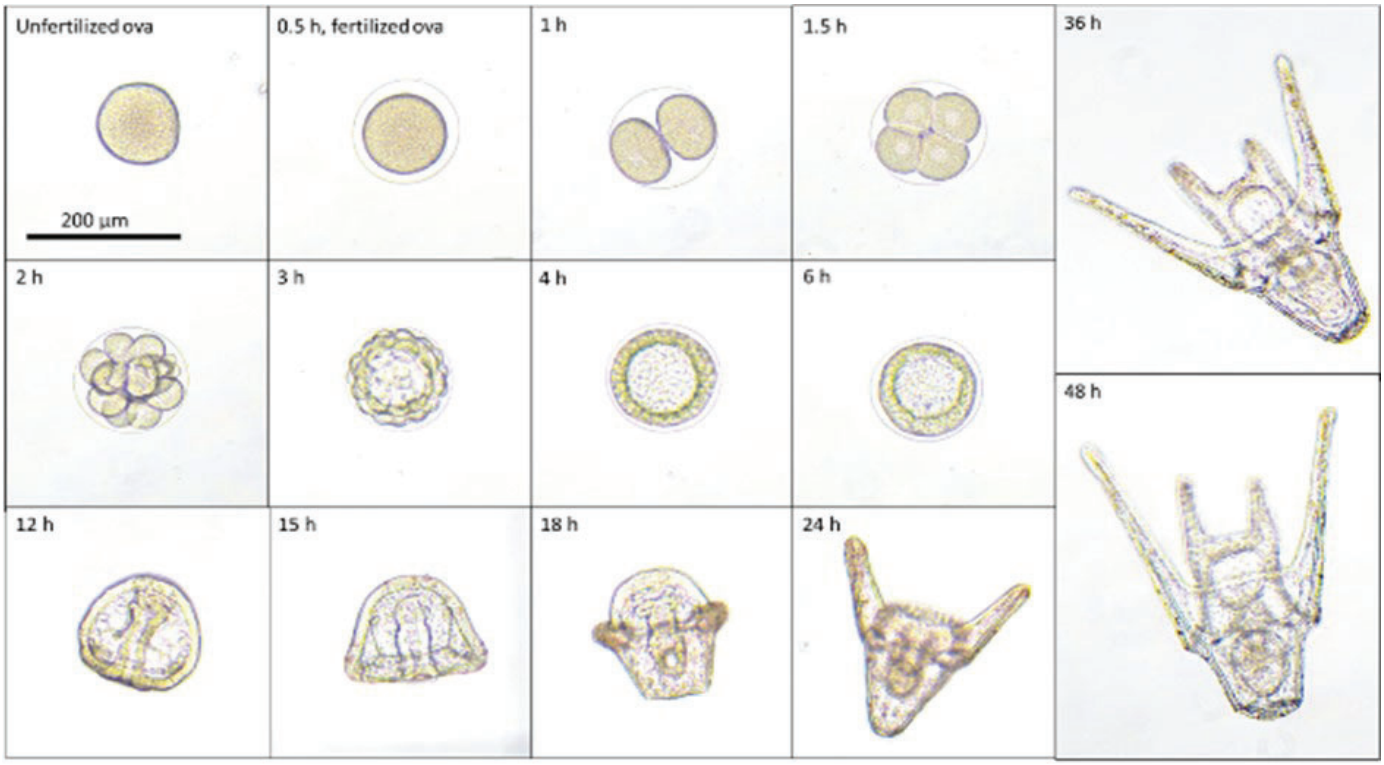

Figure 51: *Lytechinus variegatus* normal developmental schedule at 26 °C.

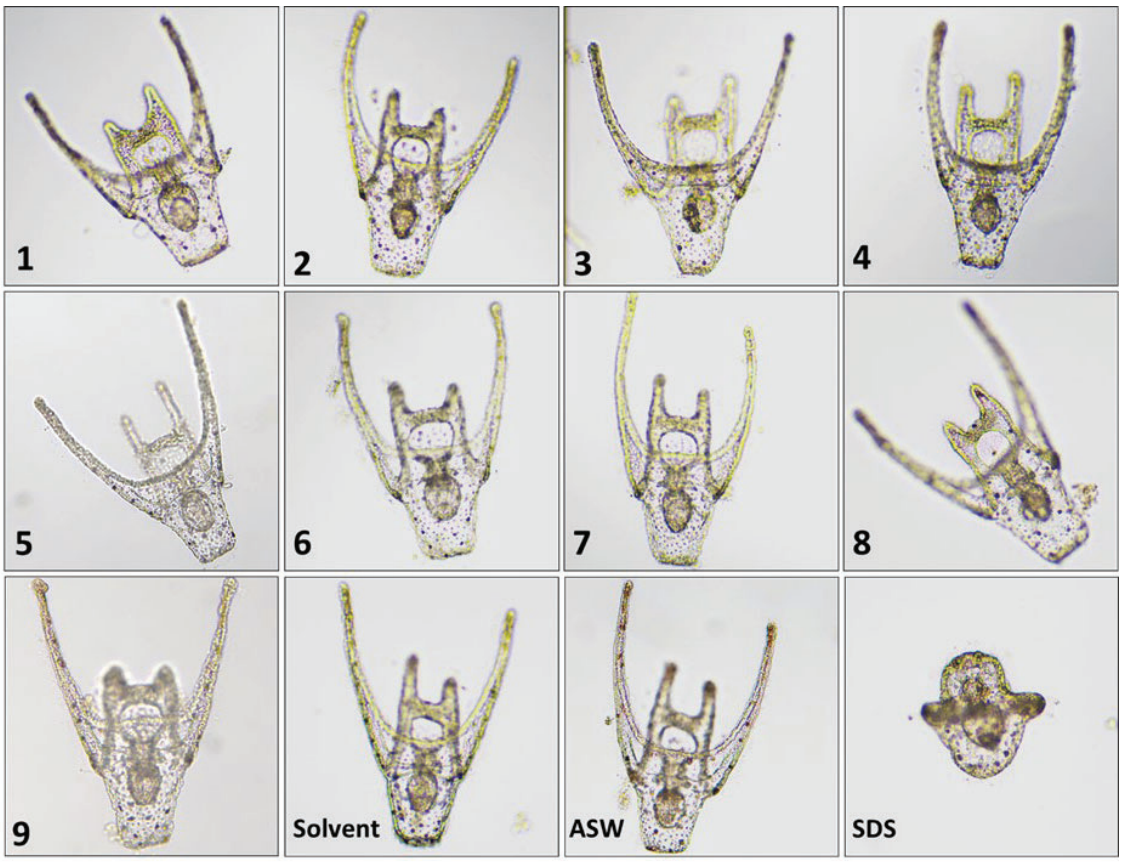

Figure 52: Typical sea urchin embryo development for Fagatele Bay water samples.

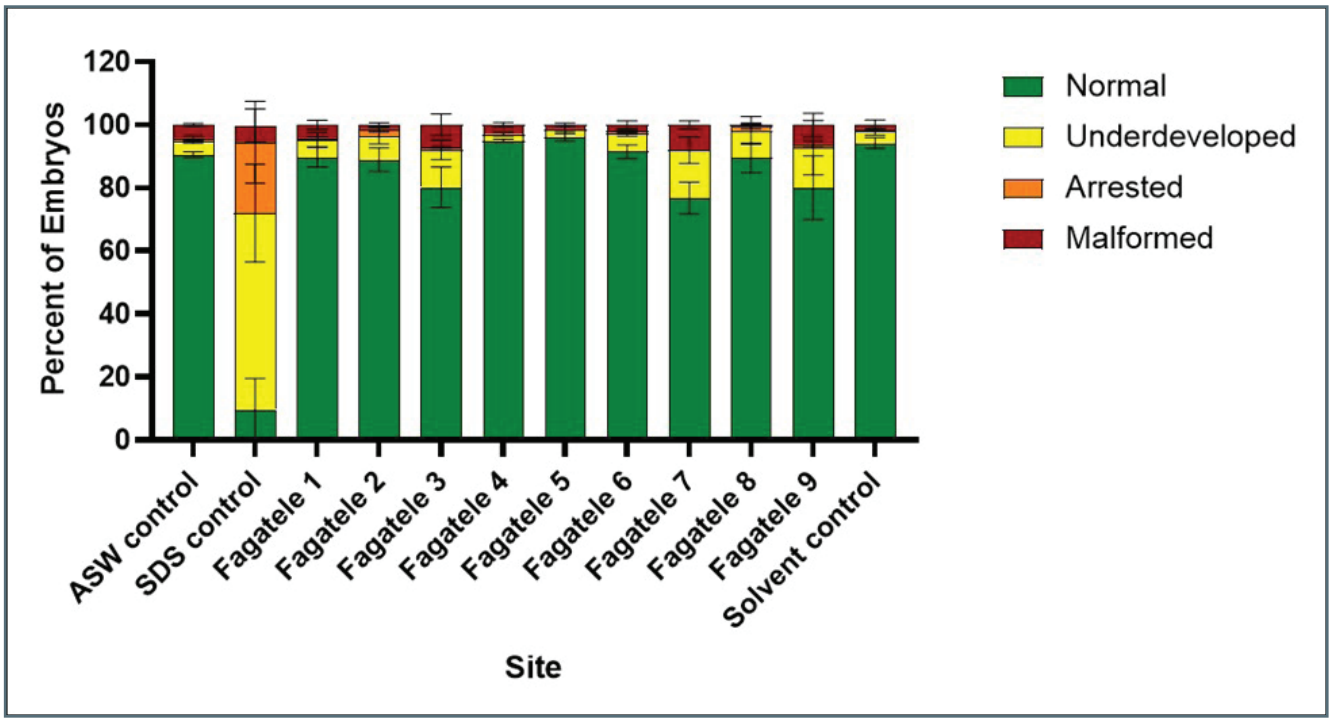

Figure 53: Proportions of sea urchin embryos at each developmental stage 48 h post-fertilization.

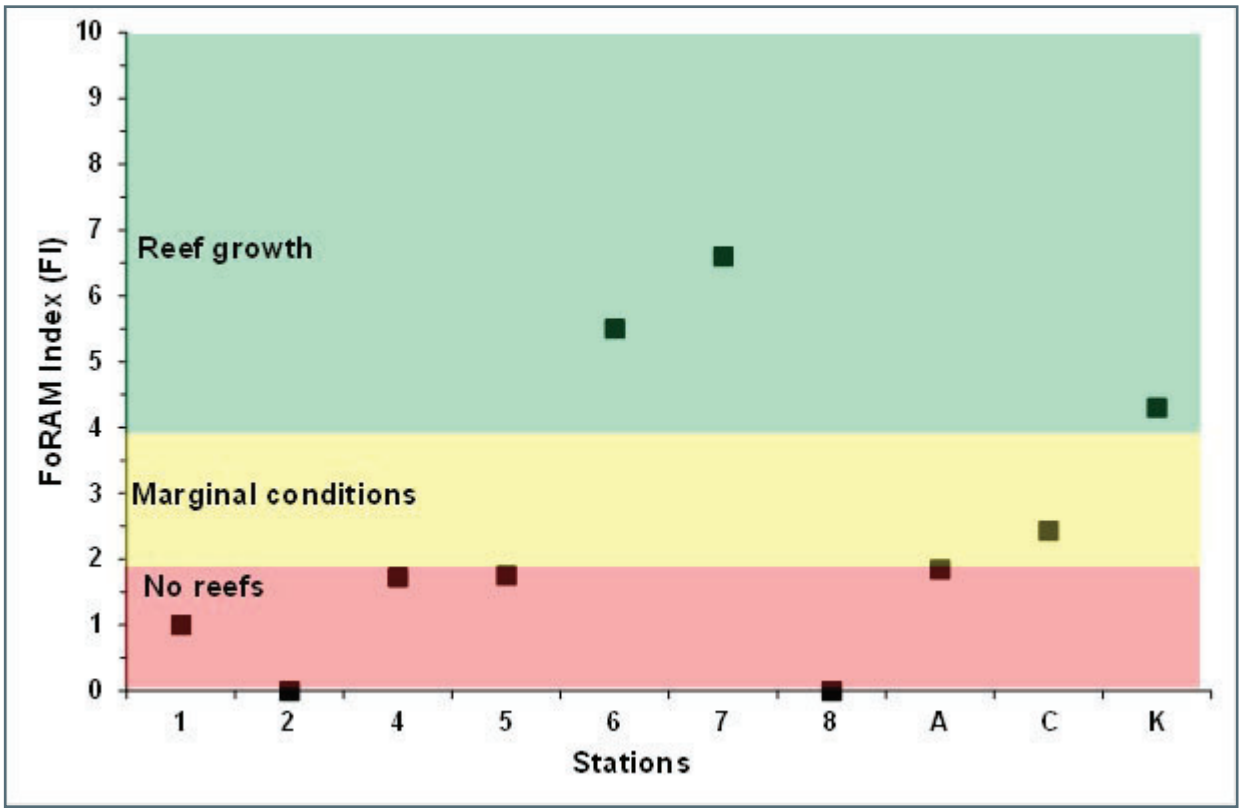

Figure 54: Foraminifers in Reef Assessment and Monitoring Index (FoRAM Index or FI) by site for Fagatele Bay.

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Appendix

Python script for enumerating colonies

```
#! /home/llhasa/anaconda3.1/bin/python3

#  Jeff Guyon
#  December 17, 2019
#  Some code derived from code from Sarthak J Shetty - see https://github.com/SarthakJShetty/Algae

#cv2 is OpenCV which will be used to carry out pixel modification
import cv2
#Importing os here to make a status_logger folder and .txt file
import os
#argparse is used to manage the input arguments
import argparse
#glob makes it so we can import a list of files from a directory
import glob

FileNumber = 0

# SET THE CURRENT WORKING DIRECTORY
#os.chdir('C:/Users/Jeff Guyon/Desktop/Python/ColonyCount-Python/')
# SET THE File Directory
PictFileLocation = 'C:/Users/Jeff Guyon/Desktop/Python/ColonyCount-Python/Tifs/LauraFiles/'

def image_viewer(image, File_Name):
    '''Instead of repetitively writing code to view image
    Function to view the file here has been written'''

    image_viewer_status_key = "[INFO] Image window is open"
    print(image_viewer_status_key)

    # resize the image to make it bigger
    LargerImage = cv2.resize(image,(1000, 720))
```

```
    winname = f"{File_Name}"
    cv2.namedWindow(winname)        # Create a named window
    cv2.moveWindow(winname, 40,30)  # Move it to (40,30)

    # display the image
    cv2.imshow(winname, LargerImage)
    while cv2.waitKey(0) != ord(' '):
        print('press spacebar to continue')

    #cv2.waitKey(0)
    cv2.destroyAllWindows()

    image_viewer_status_key = "[INFO] Image window has been closed"
    print(image_viewer_status_key)
        # return image

def contouring(ThreshType, Lower_Threshold, Center_Threshold, file_name, output_name, FileNumber):
    '''This function applies contours on the image passed to it
        The contours are applied based on co-ordinates passed to it
        from the pre_contouring() function'''

    ImageToContour=cv2.imread(file_name)
    processed_image_to_contour=cv2.resize(ImageToContour, (0,0), fx=0.25, fy=0.25)
    grayscale_image = cv2.cvtColor(processed_image_to_contour, cv2.COLOR_BGR2GRAY)

    #find the countours
    Center_ret,Center_thresh = cv2.threshold(grayscale_image,Center_Threshold,255,0)
    #contours, hierarchy = cv2.findContours(Center_thresh,cv2.RETR_TREE,cv2.CHAIN_APPROX_SIMPLE)  # only stores
certain points
    contours, hierarchy = cv2.findContours(Center_thresh,cv2.RETR_TREE,cv2.CHAIN_APPROX_NONE)

    #sort the countours by areasize - Jan 30, 2020
    areaArray = []
```

```
    for i, c in enumerate(contours):
        area = cv2.contourArea(c)
        areaArray.append(area)

    #first sort the array by area
    sorteddata = sorted(zip(areaArray, contours), key=lambda x: x[0], reverse=True)

    cnt = sorted(contours, key=cv2.contourArea)

    # height, width, number of channels in image
    height = processed_image_to_contour.shape[0]
    width = processed_image_to_contour.shape[1]
    channels = processed_image_to_contour.shape[2]
    ImageArea = height * width

    #find the nth largest contour [n-1][1]
    AreaOfContour = cv2.contourArea(sorteddata[0][1])
    if (cv2.contourArea(sorteddata[0][1]) >= (ImageArea * 0.95)):   #means the contour area is great than 95% of the image area - basically the whole pict with some error
        largestcontour = sorteddata[1][1]  # use the second largest contour as the first is the entire image
    else:
        largestcontour = sorteddata[0][1]  # use the largest contour - means the plate is intersecting with the perimeter of the image

    #find the center of the plate - second largest contour
    M = cv2.moments(largestcontour)
    cX_Center = int(M["m10"] / M["m00"])
    cY_Center = int(M["m01"] / M["m00"])



#    #find the center of the plate - largest contour
#    largest_contour = max(contours, key = cv2.contourArea)
#    M = cv2.moments(largest_contour)
#    cX_Center = int(M["m10"] / M["m00"])
#    cY_Center = int(M["m01"] / M["m00"])
```

```
#find the contours of the image
#ret,thresh = cv2.threshold(grayscale_image,Lower_Threshold,255,0)

if ThreshType == 'Absolute':
    ret,thresh = cv2.threshold(grayscale_image,Lower_Threshold,255,cv2.THRESH_BINARY )
    #image_viewer(thresh, "Standard Threshold")
elif ThreshType == 'AdaptThreshMean':
    thresh = cv2.adaptiveThreshold(grayscale_image,255,cv2.ADAPTIVE_THRESH_MEAN_C, cv2.THRESH_BINARY,51,31)
    #image_viewer(thresh, "Adaptive Threshold - Mean")
elif ThreshType == 'AdaptThreshGaussian':
    thresh = cv2.adaptiveThreshold(grayscale_image,255,cv2.ADAPTIVE_THRESH_GAUSSIAN_C, cv2.THRESH_BINARY,41,31)
    #image_viewer(thresh, "Adaptive Threshold - Gaussian")
else:
    print(f'The wrong TheshType was provided....')

#contours, hierarchy = cv2.findContours(thresh,cv2.RETR_TREE,cv2.CHAIN_APPROX_SIMPLE)
contours, hierarchy = cv2.findContours(thresh,cv2.RETR_TREE,cv2.CHAIN_APPROX_SIMPLE)

# Count the number of contours
FinalContours = []  # make an empty list
if not FinalContours:
    print(f'Empty')  # Just put in here for error correcting
NumContours = 0
Quadrant_UpperRight = 0
Quadrant_UpperLeft = 0
Quadrant_LowerRight = 0
Quadrant_LowerLeft = 0

for contour in contours:
    rect = cv2.boundingRect(contour)
    area = rect[2] * rect[3]
    M = cv2.moments(contour)
    if M["m00"]:
```

```
        cX = int(M[“m10”] / M[“m00”])
        cY = int(M[“m01”] / M[“m00”])
    else:
        cX = 0
        cY = 0
    radius = 190
    if (area < 250 ) and ((cX - cX_Center)**2 +(cY - cY_Center)**2 < radius**2):  #select contours with a small area and if located within a radius of the center
        FinalContours.append(contour)  # Put all the contours together
        NumContours = NumContours + 1  # Count the number of contours
        if ((cX - cX_Center) > 0) and ((cY - cY_Center) >= 0):
            Quadrant_LowerRight = Quadrant_LowerRight + 1
        if ((cX - cX_Center) <= 0) and ((cY - cY_Center) > 0):
            Quadrant_LowerLeft = Quadrant_LowerLeft + 1
        if ((cX - cX_Center) >= 0) and ((cY - cY_Center) < 0):
            Quadrant_UpperRight = Quadrant_UpperRight + 1
        if ((cX - cX_Center) < 0) and ((cY - cY_Center) <= 0):
            Quadrant_UpperLeft = Quadrant_UpperLeft + 1


  # print the distribution of contours to the monitor
  print(f’The total number of contours in {file_name} is {NumContours}’)
  print(f’The distribution of contours by location is:’)
  print(f’    Quadrant_UpperRight: {Quadrant_UpperRight}’)
  print(f’    Quadrant_UpperLeft:  {Quadrant_UpperLeft}’)
  print(f’    Quadrant_LowerRight: {Quadrant_LowerRight}’)
  print(f’    Quadrant_LowerLeft:  {Quadrant_LowerLeft}’)
  print(f’    ----------------------------’)
  print(f’    Total Sum:          {Quadrant_UpperRight+Quadrant_UpperLeft+Quadrant_LowerRight+Quadrant_LowerLeft}\n\n’)


  # print to a file
  # print the distribution of contours to the monitor
  f=open(output_name, “a”)
  print(f’The total number of contours in {file_name} is {NumContours}’, file=f)
  print(f’The distribution of contours by location is:’, file=f)
```

```
    print(f'    Quadrant_UpperRight: {Quadrant_UpperRight}', file=f)
    print(f'    Quadrant_UpperLeft:  {Quadrant_UpperLeft}', file=f)
    print(f'    Quadrant_LowerRight: {Quadrant_LowerRight}', file=f)
    print(f'    Quadrant_LowerLeft:  {Quadrant_LowerLeft}', file=f)
    print(f'    ----------------------------', file=f)
    print(f'    Total Sum:          {Quadrant_UpperRight+Quadrant_UpperLeft+Quadrant_LowerRight+Quadrant_LowerLeft}\n',
file=f)
    print(f'    ------------------------------------------------------------\n\n', file=f)
    f.close()  # close the file

    #print to a csv
    f=open(output_name[:-4] + '.csv', "a")
    JustFileName = os.path.basename(FileName)
    print(f'{FileNumber},{JustFileName},{Quadrant_UpperRight},{Quadrant_UpperLeft},{Quadrant_LowerRight},{Quadrant_
LowerLeft},{NumContours}', file = f)
    f.close()

    # draw the contours
    contoured_image = cv2.drawContours(processed_image_to_contour, FinalContours, -1, (0,255,0), 2)
    #draw the largest contour
    #contoured_image = cv2.drawContours(contoured_image, sorteddata[3][1], -1, (255,0,0), 2)
    #draw the largest contour
    contoured_image = cv2.drawContours(contoured_image, largestcontour, -1, (0,0,255), 2)

    # Add a point and description to designate the middle of the largest contour at the Center_Threshold
    cv2.circle(contoured_image, (cX_Center, cY_Center), 7, (255, 255, 255), -1)
    cv2.putText(contoured_image, "center", (cX_Center - 20, cY_Center - 20),
        cv2.FONT_HERSHEY_SIMPLEX, 0.5, (255, 255, 255), 2)

    # label the top left corner with the number of contours counted
    NumContoursText = f'{NumContours} Contours'
    cv2.putText(contoured_image, NumContoursText, (30, 50),
        cv2.FONT_HERSHEY_SIMPLEX, 0.5, (255, 0, 0), 2)
```

```
    # display the image
    #image_viewer(contoured_image, Lower_Threshold)

    #print the file
    #cv2.imwrite(PictFileLocation + FileName +'.png',contoured_image)
    cv2.imwrite('Analysis/' + str(FileNumber) +' - ' + JustFileName[:-4] + '-contoured.png',contoured_image)



# Main Program
# define the Center_Threshold used to find the center of the plate
Center_Threshold = 100

#FileName = ''
#parser = argparse.ArgumentParser()
#parser.add_argument("--file_name", help="image-to-analyze")
#args = parser.parse_args()
#if args.file_name:
#    FileName = args.file_name
#else:
#    print(f'Rerun and enter filename...')

# enter the filename
#FileName = input('Enter the file to quantitate: ')

# define the output file
from datetime import datetime
now = datetime.now()
output_name = "Analysis/ContourCount" + now.strftime("Date-%d-%m-%Y-Time-%H-%M-%S") + ".txt"
print(f'The following analysis was completed on {now.strftime("Date:%d-%m-%Y  Time:%H-%M-%S")}:\n\n',
file=open(output_name, "a")) # open the .txt file for the results
print(f'FileNumber, FileName, UpperRight, Upper Left, LowerRight, LowerLeft, Total Counts, {now.strftime("Date:%d-%m-%Y
Time:%H-%M-%S")}', file=open(output_name[:-4] + '.csv', "a"))
```

```
# loop through the Lower_Thresholds to find the best threshold for the contours
ThresholdMethod = 2  # can control the ThresholdType

filelist = glob.glob(PictFileLocation+"*.tif")
#Continue = 'Y'
#while (Continue == 'Y'):
#Contours are determined and applied onto the
for FileName in filelist:
    FileNumber += 1
    if ThresholdMethod == 0:  # Uses absolute values to determine thresholds, not good for when lighting changes on plate
        for Lower_Threshold in range(130, 160, 10):   # Cycle through the lower threshold limits
            contouring('Absolute', Lower_Threshold, Center_Threshold, FileName, output_name, FileNumber)
    elif ThresholdMethod == 1:  # Uses relative values to determine thresholds - cv.ADAPTIVE_THRESH_MEAN_C: The threshold value is the mean of the neighbourhood area minus the constant C.
        contouring('AdaptThreshMean', FileName, Center_Threshold, FileName, output_name, FileNumber)
    elif ThresholdMethod == 2:  # Uses relative values to determine thresholds - cv.ADAPTIVE_THRESH_GAUSSIAN_C: The threshold value is a gaussian-weighted sum of the neighbourhood values minus the constant C
        contouring('AdaptThreshGaussian', FileName, Center_Threshold, FileName, output_name, FileNumber)

#close
```

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