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A survey of environmental pollutants and cellular-stress markers of *Porites astreoides* at six sites in St. John, U.S. Virgin Islands

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Abstract Coral communities along the coast of St. John, U.S. Virgin Islands have exhibited site-specific behavior in declines. In order to determine if these specific coral communities are stressed and whether a pollutant or environmental factor present at this site is a probable stressor, we surveyed six near-shore coral communities in St. John, USVI for environmental pollutants and to determine the cellular physiological condition of the coral, *Porites astreoides*. The six sites within St. John are Cruz Bay, Caneel Bay, Hawksnest Bay, Trunk Bay, Tektite Reef in Beehive Bay, and Red Point. Red Point was considered the reference site because of its abundance and diversity of species, and it was the furthest removed from down-stream and down-current anthropogenic activities. All sites showed distinct cellular-stress marker patterns, indicating that the physiological condition of each population was different. Populations at Cruz, Hawksnest, Trunk, and Tektite were stressed, as indicated by high levels of DNA lesions and expression of stress proteins. Hawksnest and Tektite were contaminated with polyaromatic hydrocarbons (PAHs), while Cruz was contaminated with semi-volatile organochlorines and nitrogen-based biocides. At least for Hawksnest and Tektite, stress-marker patterns were consistent with an exposure to PAHs. Fecal coliform levels were high in Cruz and Trunk, indicating fecal contamination, as well as consideration for management action. Results from this study serve as a justification for a more thorough and methodical investigation into the stressors responsible for declines of coral populations within St. John. Furthermore, this study supports the argument for the importance of local factors contributing to regional coral reef declines; that not all forces impacting coral are global.

Keywords Cellular stress · Coral · Environmental pollutants · Stress-markers

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Introduction

Coral reefs are among the world's failing ecosystems, exhibiting significant rates of decline in the past 30 years (Downs et al. 2005a, b, 2006a). Coral reef degradation in the Caribbean has exemplified these recent punctuated declines, with some areas being completely depopulated while other reefs continue to show deterioration (Bellwood et al. 2004; Carpenter et al. 2008; Coelho and Manfrino 2007; Gardner et al. 2003; Rogers 2008; Szuromi 2003). Coral reefs of St. John, U.S. Virgin Islands (USVI) have not been immune to this affliction, even reefs and coral communities within the U.S. National Park have been affected (Edmunds 2002; Muller et al. 2008; Whelan et al. 2007). Decline of coral reefs and communities are not uniform across broad geographic regions. For example, a recent study focusing on coral reefs in the USVI demonstrated that the prevalence of coral mortality on near-shore reefs was almost 300% more when compared to corals in mid-shelf-island reefs (Smith et al. 2008). This same study also demonstrated that the prevalence of coral morbidity was nearly 60% more in near-shore reefs as compared to mid-shelf reefs (Smith et al. 2008). In near-shore reefs, coral reef expansion and degradation can be even more localized; coral populations in embayments or coves in adjoining watersheds may show radical differences in degradation or expansion rates (Golbuu et al. 2008; Smith et al. 2008). Such fine-scale, localized differences in expansion/degradation rates between coral populations are a reflection of the presence and type of stressors that may afflict a specific coral population.

Stressors impacting corals and coral reefs can be conveyed through three major matrixes: atmospheric, marine, and terrestrial. Atmospheric and advective-related stressors include deposition of pollutants via dust storms that are generated thousands of miles away (e.g., African dust storms), deposition of pollutants from fixed localized sources (e.g., landfill burning, industrial emissions), and transit sources, such as emission from cruise ships or air planes. Marine stressors are carried to target reefs from their sources through currents, or brought to the reef through anthropogenic routes, such as by watercraft or swimmers (Jameson et al. 2002). In island geography, watershed run-off can enter into coves and embayments through surface routes such as gullies, streams and rivers, or through sub-surface routes such as freshwater seepages (Jameson et al. 2002; Wagle 2007). Run-off from watersheds into coves and embayments can contain anthropogenic pollutants, as well as high volumes of freshwater and sediment (Harborne et al. 2006; Jameson et al. 2002; Wagle 2007). Exposure of corals to stressors from these three vectors can occur in any combination, which makes it very difficult in determining if a single stressor among a multitude of stressors was conveyed to a coral population by a specific vector.

The overarching goal of our work is to begin addressing declines in coral reefs within the vicinity of St. John, USVI as they relate to pollutants. One of the first objectives of an investigation into degrading natural resources is to establish a relationship between the relative contribution of stressors and the resulting pathology in the target populations. The next step in the investigation is to identify the source of the stressor, so that management can mitigate stressor effects by regulating source output. Before a stressor and a source can be linked, a survey must be conducted to determine the presence of pollutants at a coral site, determine whether the corals are exhibiting declines in health condition, and the nature of the disease(s). This is done to ascertain whether a pollutant or environmental factor is in fact acting as a stressor; a stressor being defined as an agent that induces a homeostatic response in an organism. The aim of our study is to take the first step in an investigative management assessment by surveying six coral reefs that are near-shore in St. John, USVI for environmental pollutants and to determine the cellular physiological condition of the coral, *Porites astreoides*. These six sites reside in embayments/coves between two and 85 m from shore that are in the direct shadow of six different watersheds. Four sites are within U.S. National Park boundaries and three of these sites have documented coral declines in the past fifteen or more years (Rogers and Miller 2006; Turgeon et al. 2002).

Materials and methods

Study sites

Six sites along the coast of St. John, U.S. Virgin Islands (Fig. 1) were examined in this study: Cruz Bay (Fig. 2a), Caneel Bay (Fig. 2b), Hawksnest Bay (Fig. 2c), Trunk Bay (Fig. 2d), Tektite Reef (Fig. 2e) and Red Point (Fig. 2f). Cruz Bay is a harbor area for recreational crafts, ferries, and is the primary receiver for pollutants come from the town of Cruz Bay (Fig. 2a). Caneel Bay lies within the U.S. Virgin Islands National Park whose watershed is dominated by Caneel Bay Resort (Fig. 2b). Hawksnest Bay is within the U.S. National Park and is a tourist beach and swimming attraction (Fig. 2c). Trunk Bay is within the U.S. Virgin Islands National Park and is a tourist beach and swimming attraction (Fig. 2d). Tektite Reef is adjacent to Great Lameshur Bay, resides within the U.S. Virgin Islands National Park, and is accessed only by recreational boat (Fig. 2e). Red point was chosen as a potential reference site because it had minimal anthropogenic intrusion from

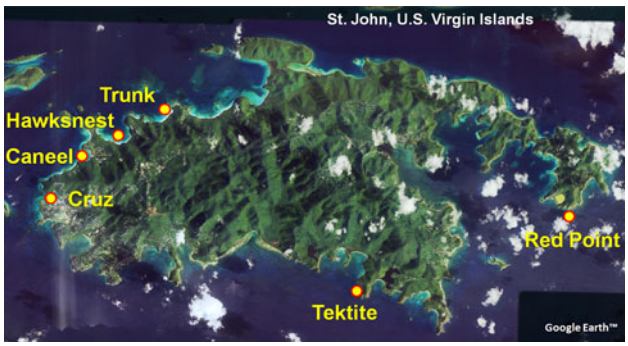


Fig. 1 Collection sites on St. John Island, U.S. Virgin Islands. Coral, sediment and water samples were collected from six sites along the coast of St. John

swimmers, boats, and land-based run-off; it lies beyond U.S. Virgin Islands National Park boundaries (Fig. 2f).

Coral biopsy sampling

At each site, *P. astreoides* colonies were sampled at a depth between 3–5 m. Each colony had a surface area between 150 and 300 cm^2. A single biopsy plug (1.5 cm diameter $\times$ 8 mm deep) was collected from each colony; six colonies per site were sampled. The plugs were placed in opaque, 35 mm film canisters and immediately frozen in liquid nitrogen and stored at $-80°C$ until analysis.

Fig. 2 Watershed sites on St. John Island, U.S. Virgin Islands indicating where sediment samples were collected. **a** Cruz Bay, **b** Caneel Bay, **c** Hawksnest Bay, **d** Trunk Bay, **e** Tektite Reef (Beehive Bay) and **f** Red Point. *Numbers* in each panel indicate the identification of each sediment sample collected, while the adjacent *dot* indicates the position where the sediment was sampled. In **e**, sample #2 was collected within 3 m of the mooring anchor

Enzyme-linked immuno-sorbent assay (ELISA) analysis

Samples were assayed according to methods adapted from Downs et al. (2006a, b). *Porites* biopsy disks were separately ground frozen to a fine powder in a pre-chilled mortar and pestle using liquid nitrogen. About 100 µl of frozen sample powder was placed in locking 1.8 ml Eppendorf microcentrifuge tubes along with 1400 µl of a denaturing buffer consisting of 2% SDS, 50 mM Tris-HCl (pH 7.8), 15 mM dithiothreitol, 10 mM ethylene diaminetetraacetic acid (EDTA), 3% polyvinylpolypyrrolidone (PVPP) (wt/vol), 0.005 mM salicylic acid, 0.001% (v/v) dimethyl sulfoxide, 0.01 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 0.04 mM bestatin, 0.001 E-64, 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamidine, 5 µM a-amino-caproic acid, and 1 µg per 100 µl pepstatin A. Samples were vortexed for 15 s, heated at 93°C for 6 min, with occasional vortexing, and then incubated at 25°C for 10 min. Samples were subject to centrifugation (13,500×*g* for 8–10 min) and the middle-phase supernatant was aspirated and placed in a new tube. The sample supernatant was subjected to a protein concentration assay by the method of Ghosh et al. (1988).

Antibodies used in this study were generated in rabbits against synthetic peptide antigens conjugated to ova albumin. Antigens were designed based on conserved sequences found in cnidaria, including *Acropora millepora, P. astreoides, Acropora palmata, Montastrea annularis* (http://sequoia.ucmerced.edu/SymBioSys/), and *Nematostella vectensis* (http://genome.jgi-psf.org/Nemve1/Nemve1.home.html). All antibodies are mono-specific polyclonal antibodies made against a synthetic residue polypeptide that reflects a specific and unique region of the target protein. Antigens are designed based on a highly conserved, though unique, domain of the target protein that exhibited a high predicted immunogenicity index. Antibodies were tested for titer and specificity against antigen based on a method described in Crowther (2001).

One-dimensional SDS-PAGE and western blotting were used to optimize the separation of target proteins and validate the use of specific antibodies for *P. astreoides* protein extracts (Downs et al. 2006a, b). Total soluble protein (15–40 µg) from three randomly chosen samples was electrophoresed in a 12.5% SDS-PAGE preparative gel containing 0.001 M TCEP (*tris*(2-carboxyethyl)phosphine; neutral pH) until the bromophenol blue dye front was near the bottom of the gel. All gels were blotted onto PVDF membranes (Millipore) using a wet transfer system. The membranes were blocked in 5% non-fat dry milk, and assayed with primary antibody for 1 h at 4°C. The blots were then washed in TBS four times, and incubated in a 1:30,000 dilution of alkaline phosphatase-conjugated donkey anti-rabbit Fab fragment solution (Jackson ImmunoResearch Laboratories, Westport, PA) for 1 h at 25°C. Blots were washed again four times in TBS, and developed using Sigma Fast NBT/BCIP solution. To characterize potential artifacts resulting from antibody non-specific cross-reactivity, blots were over-developed for at least seven min. Calibration using a quantitative standard showed that 0.05 attomole of target protein can be detected at this level of sensitivity.

Once validated, antibodies and samples were optimized for ELISA using 96-well microplates in an 8 × 3 × 3 factorial design (Crowther 2001). Twenty-five micrograms of sample was placed in a 96-well Nunc Maxisorp (white) microplate. Each sample was assayed in triplicate. An eight-point calibrant standard curve was plated in triplicate for each microplate. Calibrant was the antigen used to produce the antibody. Samples were incubated for 12 h at 25°C, and then aspirated from the microplate. All wells were blocked with blocking buffer containing 50 mM TBS and 5% non-fat dry milk for 1 h. Blocking buffer was aspirated, and a 65 µl primary antibody solution (1:10,000 titer) was added to each well for 1 h. Wells were washed four times with TBS using a Bio-Tek EL-404 microplate washer. Each well was then incubated for 1 h with a secondary antibody solution consisting of 1:30,000 titer HRP (horseradish peroxidase)-conjugated goat anti-rabbit secondary antibody in TBS. After the incubation, wells were washed four times with TBS as above. A chemiluminescence solution of 60 µl was added to each well (NEN Enhanced Western Lightning Luminol chemiluminescence) and the microplate read using the luminescent setting on a Bio-Tek ELx808 microplate reader.

Sample extracts were assayed using the following antibodies and Envirtue catalogue numbers: cnirdarian anti-GRP75 (cat. #AB-129), cnidarian anti-heat-shock protein 60 (Cat. #AB-1508), cnidarian anti-small heat shock protein (Cat. #AB-H105), cnidarian anti-ferrochetalase (Cat. #AB-FC-1939), cnidarian anti-cytochrome P450 1-class homologue (Cat. #AB-Cyp1), cnidarian anti-MutY (cat.#ABMUTY), cnidarian anti-GST (Cat. #AB-GST-CN), cnidarian anti-heme oxygenase I (Cat. #AB-HO-1944), anti-ubiquitin (Cat. #AB-U100) and anti-MXR (ABC family of proteins). All samples were assayed individually and in triplicate; intra-specific variation of less than 13% was achieved throughout the 96-wells of each microplate. A calibrant relevant to a given antibody was plated in sextuplicate on each respective plate assayed to construct an eight-point calibration curve.

DNA AP site assay

Sample DNA was obtained using the Get*Pur* DNA Purification kit (Dojindo Molecular Technologies, Gaithersburg, MD) with minor modifications. Briefly, 50–60 mg of

frozen (−80°C) ground coral tissue powder were placed in a 1.5 ml tube containing 400 µl lysis buffer and approximately 10 mg polyvinylpolypyrrolidone (PVPP, Sigma) for removal of any polyphenolic compounds associated with the sample. The samples were vortexed for 5 s, 10 µl of proteinase K solution was added, and the samples vortexed. This mixture was heated at 65°C for 10 min, removed from heat and cooled for 2 min. A 2 µl aliquot of RNase solution was added. The tubes were vortexed quickly and incubated at room temperature for 2 min. Eighty microliters of Solution I and Solution II (supplied with the kit) were added and the tubes were vortexed after each addition. The samples were centrifuged at 14,000 rcf at 4°C for 10 min. The supernatant was aspirated and placed in a clean tube. An equal volume of 100% ethanol (Pharmco-Aaper, Brookfield, CT) was added and the samples vortexed, then centrifuged again as above. The supernatant was decanted and the nucleic acid pellets washed with 1.0 ml of 70% ethanol. Following a final centrifugation (14,000 rcf, 5 min), the liquid was decanted and the pellets dried in a speedvac. Sample DNA was resuspended in 100 µl of 10 mM Tris-1 mM EDTA (TE), pH 8.0 and placed at 4°C overnight. DNA concentration was determined using the Quant-iT DNA HS assay kit (Molecular Probes, Eugene, OR), with recovery between 20 and 100 µg/ml.

A DNA Damage Quantification kit (Dojindo Molecular Technologies, Gaithersburg, MD) was used to determine the number of aldehyde reactive abasic sites in each nucleic acid sample with a substrate reporter substitution to increase assay sensitivity. Ten microliters of a 10 µg/ml solution was placed in a 0.5 ml tube and 5 µl of aldehyde reactive probe (ARP) solution was added. The manufacturer's protocol for the remainder of the labeling reaction was used. Samples were diluted (90 µl in 310 µl TE) and 60 µl pipetted into wells of a white polystyrene microtiter plate in triplicate, along with kit-supplied AP standards. Once DNA binding and HRP treatment were complete (including washing), 100 µl of a 50:50 mixture of luminol:oxidizer from the Western Lightning Chemiluminescent Reagent Plus kit (Perkin-Elmer, Waltham, MA) was added to each well, replacing the colorimetric substrate supplied in the kit. The plate was read immediately on a Bio-Tek Synergy HT multi-detection microplate reader set for luminescence at sensitivity settings of 150 and 180. For samples with AP site values above the range of the kit standards, dilutions were made in the TE solution, the samples were relabeled with ARP, and then reevaluated.

Porphyrin microplate fluorescence assay

Frozen ground tissue samples were prepared for porphyrin concentration determination by first aliquoting 2 mM PMSF, 2 mM benzamidine, 5 µM a-aminocaproic acid, and Protease Inhibitor cocktail (Sigma cat # P9599; 0.04 mM Bestatin, 0.001 mM E-641, µg/100 µl pepstatin A, AEBSF, leupeptin, 1,10 phenanthroline) into the inside caps of 1.5 ml locking Eppendorf microcentrifuge tubes, and then adding 150–200 µl of the frozen, ground tissue into each tube. The denaturing buffer comprised of 50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 25 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.05 mM desferioxamine mesylate, 0.001 mM sorbitol, 1% DMSO, and 4% PVPP was added to the ground tissue and the samples were vortexed for 30 s, gently inverted repeatedly for 1 min, and then incubated at 90°C for 3 min, vortexed for 30 s and then incubated again at 90°C for at least another 5 min before centrifugation. Samples were centrifuged at $20{,}800 \times g$ for 15 min and the middle phase aspirated and aliquoted into amber 1.5 ml centrifuge tubes (Fisher Scientific). Protein concentration was determined using the method by Ghosh et al. (1988).

Protoporphyrin IX (P562-9 1 g) obtained from Frontier Scientific Inc. (Logan, UT USA) was reconstituted in 12 N hydrochloric acid (HCl) (1 mg/1 ml), vortexed for 1 min and diluted to 141.7 nmol/ml of Protoporphyrin IX with 1 N HCl. The Protoporphyrin IX standard was made using 100 µl of the stock (141.7 nmol/ml) added to 900 µl of 1 N HCl for a concentration of 14.16 nmol/ml and from this stock 100 µl was further diluted to 28.333 ml in 1 N HCl. This working stock was used to create the 8-point calibrant standard curve with the highest concentration point being 5,000 fmoles and the lowest concentration at 500 fmoles. The standards and samples were diluted with a solution of 50 mM Tris-HCl (pH 7.8), 10 mM EDTA and 2% SDS before they were plated in triplicate on a black 96-well optical bottom plate (Nalge Nunc International). After plating the standards and samples (311 µl mixture), 39 µl of 9 N HCl was added to all standards and samples (total volume 350 µl) and incubated for 15 min. Plates were read on a Bio-tek HT Synergy microplate reader (Winooski, VT USA) using a 400 nm/30 excitation filter and 600 nm/40 emission filter. Photomultiplier sensitivity was optimized the strongest fluorescence signal without saturation.

Microbiology sampling and analysis

"Waters" of the U.S. Virgin Islands are defined Title 12, Chapter 7, Section 182(f) of the Virgin Islands Code (USVI 2001). USVI waters are classified into three (3) groups based on designated uses: Class A, B and C. Water samples for microbiology were collected from Trunk Bay (Class A), Cruz Bay (Class B) and Caneel (Class B).

Optimal sampling times for maximal detection of *E. coli* and total coliform (TC) input was determined by sampling throughout a tidal cycle at Cruz Bay, a known impacted site. Samples were taken in duplicate, 1 h after high tide by

beach entry to approximately 1 m water depth and submerging collection bottles approximately 0.5 m below the surface for *E. coli*, TC and fecal coliform (FC) determination. Plastic Nalgene bottles (1L) were sterilized with isopropanol and rinsed three times with sampling water before collection. Samples were transported to the lab in a cooler and processed within 6 h of collection.

Fecal coliforms (FC), total coliforms (TC) and *E.coli* were determined with membrane filtration (0.45 µm pore size, 47 mm diameter, cellulose nitrate, Millipore) (ISO 9308-1). mFC agar (Difco) (Geldreich et al. 1965) and ChromoCult® coliform agar (Merck, VWR, imported, not available in US) were used respectively and prepared according to the manufacturer's instructions. Fecal coliform samples were filtered in triplicate, applying at least two different dilutions. Plates were incubated in a waterbath at 44.5°C (American Public Health Association 1998; International Organization for Standardization 2000; US EPA 2002). After incubation for 24 h dark blue colonies were counted as FC.

Total coliforms and *E.coli* samples were filtered in quadruplicates due to the large differences in TC and *E.coli* numbers. Plates were incubated at 37°C in a waterbath for 24 h (US EPA 2002, standard method 9223). Dark blue to violet colonies were counted as presumptive *E.coli*, salmon to red colonies including blue colonies were counted as TC. *E.coli* colonies were not verified with the optional indole test; therefore counts are regarded as presumptive *E.coli*.

Total Direct Counts (TDC) of bacteria in seawater were determined from fixed seawater samples. Samples were diluted 4:1 (seawater:Z-fix) in zinc-buffered formalin (Z-Fix, Anatech Ltd., Battle Creek, MI) and held at room temperature until staining and enumeration. The acridine orange (AO) method of Hobbie et al. (1977)was used to quantify the bacteria. Briefly, 2 ml of the preserved water sample was mixed with 1 ml of 0.1 g/1000 ml AO solution (BD & Co., Sparks, MD) and added to a 15 ml glass filter filtration unit (25 mm diameter) using a 0.2 µm black polycarbonate 25 mm membrane filter (GTBP02500, Millipore, Billerica, MD) and allowed to stain under low light for 3 min. While staining, a cover slip was prepared by placing it atop a small drop of immersion oil (Type FF, Cargille, Cedar Grove, NJ) situated in the middle of a labeled glass microscope slide. After staining 3 min, the water sample was filtered at a low vacuum pressure (5–10 mm Hg). Just before the meniscus reached the surface of the filter, 2 ml sterile seawater was added as a rinse and repeated once. After the sample was completely filtered, the filter was removed from the tower (still under pressure), using forceps, and placed on the slide (cover slip removed) atop the emersion oil, using care not to create air bubbles. The cover slip was then placed on top of the filter, carefully applying pressure with the forceps to eliminate bubbles. Slides were stored frozen before viewing. Slides were viewed using a Nikon Eclipse E600 fluorescence microscope (Nikon Instruments, Melville, NY) fitted with a Nikon blue excitation filter B-2A: excitation filter 450-490 nm, dichromatic mirror (DM) 500 nm, and barrier filter (BA) 515 nm. Bacteria were counted using an ocular grid, where 20 grids or 200 cells were counted. The number of grids per filter (17671.46) was found by dividing the area of the grid (0.01 mm^2) by the area of the filter (176.7 mm^2). Bacteria per ml were calculated by dividing the total number of cells counted by the number of grids viewed. This number was then multiplied by the number of grids per filter (17671.46, shown above) to give cells per filter. Finally, cells per ml were determined by dividing cells per filter by ml of sample per filter (1.6 ml of seawater prior to fixing).

Marine sediment contaminant chemistry analysis

Three samples from each site were collected from the top 8 cm (11 cm length × 3 cm diameter) of surface sediment using a PFE-Teflon sediment tube-trap. Each tube-trap was washed with a laboratory detergent then washed with four rinses of acetone, dried, then sealed in Teflon-coated aluminum foil. Sediment from the trap was transferred to a Welch Fluorcarbon PFA-Telfon bag, sealed, and frozen until extraction.

Pesticide grade solvents were used for the extraction solvents (Fisher Scientific). Analytical standards were purchased from Ultra Scientific, RI USA. Two stock standards were prepared based on the analytes being examined. The first was a semi-volatile organochlorine pesticide stock (SVOC) which included a semi-volatile mixture (SVM-525) and an organochlorine pesticide mixture (PPM-525E) (Table 2 for list of analytes examined). The second was an NP stock which included nitrogen/phosphorous pesticide mixture 1 (NPM-525C) and nitrogen/phosphorous pesticide mixture 2 (NPM-525B). The internal standard solution (ISM-510) was used for GC–MS quantification. Sodium sulfate (Fisher Scientific) was baked at 200°C overnight and then pre-rinsed with hexane before use as part of the clean-up in EPA Method 8140.

Marine sediment samples were extracted using an ASE 200 accelerated solvent extraction system (Dionex Corporation, Sunnyvale, CA, USA). Approximately 10 g wet weight of marine sediment was placed in a hexane-rinsed aluminum dish, and diatomaceous earth (Dionex Corporation, Sunnyvale, CA, USA) was added and ground until the mixture was dry. This mixture was added to a 22 ml ASE extraction cell. Blank and spike (5 µg/g) samples were prepared in Ottawa Sand (mesh 200–300, Fisher

Scientific) and diatomaceous earth. The ASE extraction solvent was dichloromethane:acetone (1:1), run at 1500 psi and 100°C for 5 min with a flush of 50% of the ASE cell volume and a nitrogen purge for 80 s. The extract was solvent exchanged to hexane and dried to 1 ml.

A Florisil cleanup was used following USEPA method 3620b (1996). The Florisil SPE cartridge (1 g, 6 ml, Varian Inc.) was conditioned with 5 ml hexane, kept wet, and then the sample was added and collected in a 20 ml tube. The Florisil was eluted with: 3 ml hexane, 6 ml mixture (1:4 dichloromethane:hexane), 6 ml mixture (1:9 acetone:hexane). The extract was solvent exchanged and dried to 1 ml dichloromethane, internal standards were added (acenaphthene-d10, phenanthrene-d10, and chrysene-d12) and the extract was analyzed by GC–MS.

Two separate GC–MS methods were developed which included a semi-volatile/organochlorine (SVOC) method and a nitrogen/phosphorous (NP) method. Samples were run on a Varian 3800 gas chromatograph with a Saturn 2200 ion trap mass spectrometer (Varian Inc. Walnut Creek, CA, USA). The gas chromatograph was equipped with a 30 m VF-5 ms column (0.25 mm i.d., 0.25 mm film) run at 1.1 ml/min helium with a pressure pulse of 45 psi for 0.8 min. The oven temperature started at 70°C with a 1 min hold, then increased to 300°C at 4°C/min and held at 300°C for 2 min. A 1 µl sample was injected split/splitless at 250°C using a Varian CP-8400 autosampler. The transfer line temperature was 270°C, the trap was 200°C and the manifold was maintained at 80°C. The mass spectrometer was run in full scan mode. Data were analyzed using Saturn GC–MS Workstation version 6.42. A five point standard curve was run on the GC–MS for all compounds (0.01–10 µg/ml) which had a correlation coefficient greater than 0.99 with less than 15% standard deviation.

Statistics

Data were tested for normality using the Kolmogorov–Smirnov test (with Lilliefors' correction) and for equal variance using the Levene Median test. If the data were normally distributed and homogeneous, a one-way analysis of variance (ANOVA) was employed. When data did not meet the homogeneity of variances requirement for one-way ANOVA, we instead used a Kruskal–Wallis One-Way Analysis on Ranks. When significant differences were found among treatment means, we used the Tukey–Kramer Honestly Significant Difference (HSD) method, the Dunn's post-hoc test, or the Holm–Sidak test as an exact alpha-level test to determine differences between each of the populations.

Canonical correlation analysis (CCA) was used to determine the relationship and the amount of variance shared between two sets of variables, i.e., biomarker responses vs. cohorts. Canonical correlation analysis is an eigen-analysis method that reveals the basic relationships between two matrices (Gauch 1985) in our case those of the six coral populations and cellular diagnostic marker data. The CCA provided an objective statistical tool for (1) determining if populations are different from one another using sets of cellular markers that are indicative of a cellular process (e.g., protein metabolic condition, xenobiotic response), and (2) which cellular diagnostic markers contributed to those differences. This analysis required combining data from all five treatments into one matrix, which we did by expressing cellular marker responses in a given treatment as a proportion of their mean levels. Two assumptions of CCA, that stressor gradients were independent and linear, were constraints of the experimental design.

Results

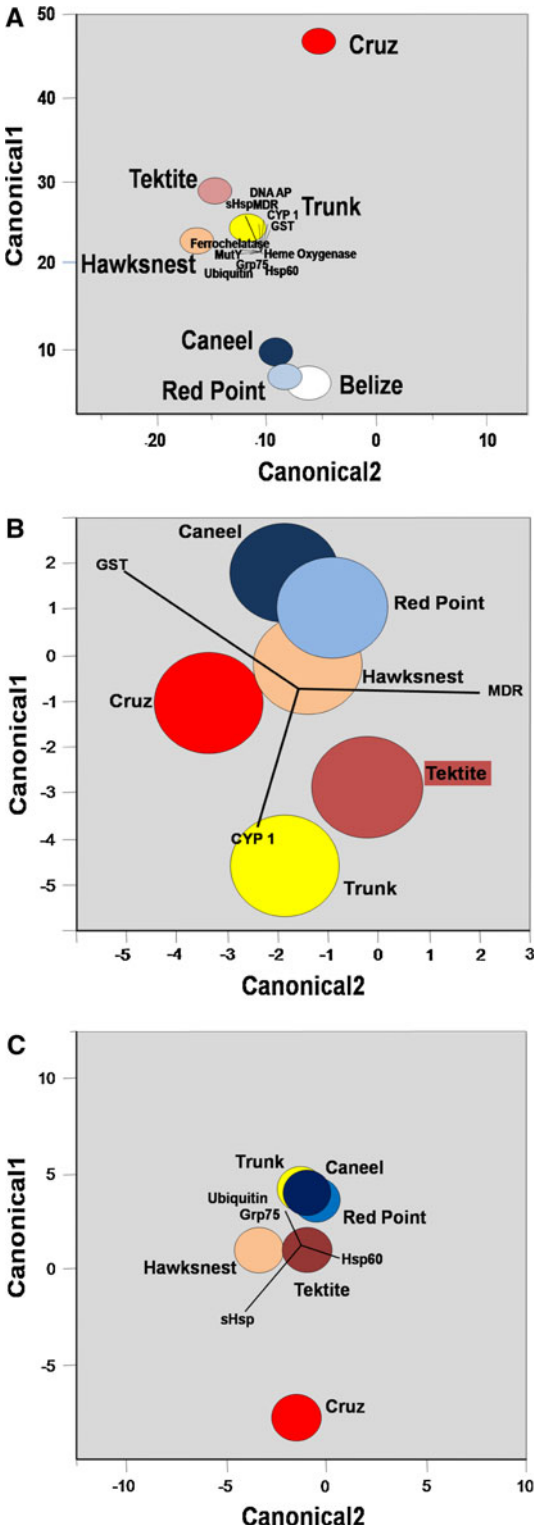
ELISA results

Health condition

Figure 3a addresses the primary question, "Do the six coral populations differ in the expression of the ten cellular-stress markers?" Two sites are correlated, Caneel and Red Point, indicating that the marker expression patterns are similar. Centroids of Hawksnest, Trunk Bay, Tektite, and Cruz Bay do not overlap, indicating that the cellular marker patterns are significantly different among these four sites both from one another, and from the Caneel and Red Point populations. The functional role of each cellular diagnostic marker is described in Table 1.

Genomic integrity

MutY is a DNA-repair glycosylase it measures the homeostatic status of DNA integrity—if DNA is being damaged this protein will be up-regulated. Levels of MutY were not significantly different among Red Point, Caneel, and Trunk (Fig. 4). Tektite and Hawksnest had more than double the amount of MutY compared to Red Point, Caneel, and Trunk Bay ($p < 0.001$; ANOVA, Holm–Sidak post hoc test), while Cruz Bay had over triple the amount of MutY as compared to Red Point. DNA apyrimidic/apurinic (DNA AP) sites are DNA damage lesions. Red Point and Caneel had significantly lower levels of DNA AP sites compared to the other four populations (Fig. 4; $p < 0.001$; ANOVA, Holm–Sidak post hoc test). Cruz Bay had almost six times the amount of DNA AP sites compared to Red Point.



◀ **Fig. 3** Canonical Centroid Plots. Original variates were cellular diagnostic marker levels expressed as a percentage of the mean value of each population. *Circles* show the 95% confidence intervals around the distribution centroid of each stressor. Biplot rays radiating from the grand mean show directions of original cellular diagnostic marker responses in canonical space. Overlapping centroids indicate that those populations are significantly correlated with one another, while non-overlapping centroids indicate are not significantly correlated ($p < 0.05$). **a** Canonical centroid plot of all cellular diagnostic markers combined from all six populations. **b** Canonical centroid plot of xenobiotic response markers from all six populations. **c** Canonical centroid plot of protein chaperoning and degradation markers from all six populations

Xenobiotic response

Canonical correlation analysis indicated that the pattern of Xenobiotic Response markers for Red Point, Caneel, and Hawksnest were correlated, while Cruz Bay, Tektite and Trunk Bay were not (Fig. 3b). A homologue to Cytochrome P450 class 1 (CYP P450) was significantly lower in the Red Point and Caneel sites compared to the other four sites (Fig. 5; $p < 0.001$; Kruskal–Wallis ANOVA, Student–Newman–Keuls post hoc test). Trunk Bay, Cruz Bay, and Tektite populations had more than double the amount of CYP P450 found in the population at Hawksnest. Red Point had significantly lower levels of glutathione-*S*-transferase (GST) compared to the other five sites (Fig. 5; $p = 0.003$; Kruskal–Wallis ANOVA, Student–Newman–Keuls post hoc test). Cruz Bay had over four times more GST than Red Point, and at least double the amount of GST compared to the other four sites. Multi-xenobiotic Response Protein (MXR; P-glycoprotein 180) was significantly lower in Red Point compared to the other five sites (Fig. 5; $p < 0.001$; Kruskal–Wallis ANOVA, Student–Newman–Keuls post hoc test). Again, Cruz Bay had the significantly highest level of MXR expression among all six sites, having almost seven times higher levels as compared to Red Point.

Porphyrin metabolism

Red Point, Caneel, Trunk Bay, and Cruz Bay had significantly lower ferrochelatase levels compared to the remaining two populations (Fig. 6; $p < 0.001$; ANOVA, Holm–Sidak post hoc test). Trunk Bay, Cruz Bay, Tektite, and Hawksnest had significantly higher levels of heme oxygenase I levels compare to Red Point and Caneel (Fig. 6; $p < 0.001$; ANOVA, Holm–Sidak post hoc test). Porphyria species exhibited such high variance among all the sites that there were no significant differences (Fig. 6).

Protein chaperoning and degradation

Canonical correlation analysis indicated that the pattern of Protein Chaperoning and Degradation markers for Red Point, Caneel, and Trunk Bay were correlated, while Hawksnest and Tektite were correlated (Fig. 3c). Mitochondrial glucose-regulated protein 75 (GRP75) levels were not significantly different among the coral populations

Table 1 Physiological functional roles of cellular diagnostic markers

Cellular parameter	Biological significance
Genomic integrity	
Mut Y	Enzymatic first step in the base excision repair pathway of OG:A; adenine glycosylase initiates removal of undamaged adenine by hydrolysis of N-glycosidic bonds forming a DNA-AP site. Elevated MutY can be caused by elevated numbers of DNA AP sites (Au et al. 1989)
DNA AP	DNA interacting with reactive oxygen species (ROS) is a major cause of DNA damage. In particular, the hydroxy radical generated from superoxide and hydrogen peroxide by the Fenton reaction. One of the lesions resulting from oxidative hydroxy radical damage on the deoxyribose moiety will lead to the release of free bases from DNA, generating strand breaks with various sugar modifications and simple AP sites (Kow et al. 1991; Kow 1994)
Xenobiotic response	
Cytochrome P450 Class 1	Hemoprotein involved in oxidative stress. Oxidation of xenobiotic often allows sulfonation or glutathionation by cellular enzymes (Downs et al. 2005a)
Glutathione-*S*-transferase	GSTs usually are associated with detoxification by conjugation of genotoxic and cytotoxic xenobiotic electrophiles derived from drugs, carcinogens, and environmental pollutants. During a xenobiotic challenge, glutathione can be conjugated to a xenobiotic by GST, representing a major detoxification pathway (Sies 1999). Additionally, GST detoxifies DNA hydroperoxides, playing an important role in DNA repair
MXR	*P*-glycoprotein members of a superfamily of proteins that act as channels and transporters of solutes across membranes (Downs 2005). They play a role in xenobiotic detoxification. Currently, it is believed that *P*-glycoproteins effectively process certain xenobiotics to exit the cell
Porphyrin metabolism	
Ferrochelatase	An enzyme that inserts ferrous iron into protoporphyrin IX to form heme. In invertebrates, it is able to utilize several different metals and porphyrin substrates (Thunell 2000). Cellular detoxification pathways and essential cellular metabolism require heme or porphyrin-based substrates. If an organism is going to up-regulate metabolic or xenobiotic detoxification pathways, it will need to increase its heme production, and up-regulate ferrochelatase
Heme oxygenase	HO (also Hsp32) is up-regulated in response to oxidative damage. Heme oxygenase degrades heme to biliverdin, free iron, and carbon monoxide (Thunell 2000)
Porphyrin	Macrocyclic compounds of the heme biosynthetic pathway and ubiquitous in nature, forming the basic structure of hemoproteins, e.g., chlorophyll, hemoglobin; cytochromes and catalase. Porphyrins accumulate in its metabolic pathway (Marks 1985). Agents such as xenobiotics e.g., pesticides, PCBs, PAHs can inhibit enzymes in this pathway
Protein chaperoning & degradation	
Hsp 60 and Grp75	Essential components for cellular function, during both normal and stressed conditions. They regulate protein structure and function under normal physiological conditions as well as during and following stress by renaturing denatured proteins into active states in an ATP-dependent manner. Hsp60 and Grp75 concentrations increase in response to stress, specifically in response to increased protein synthesis and denaturation. Grp75 helps shuttle nascent proteins and is crucial to their maturation (Ellis 1996; Hartl 1996)
Ubiquitin	A protein found in most phyla, used in marking proteins for rapid degradation. Increased concentrations are an indication of increased protein degradation, and hence increased protein turnover. The level of ubiquitin is an index of the structural integrity of the protein component of the superstructure of the cell (Hartl 1996; Downs 2005)
Small heat shock protein (sHSP)	(sHsp = total small heat-shock protein isoforms)—Small Hsps from all phyla share a common motif near the carboxyl-terminal end of the protein, known as the "heat-shock domain" or α-crystallin domain. These proteins are not expressed in adult coral under nominal condition. It is only during dire, stressful conditions that these proteins are expressed, usually in response to metabolic failure. These proteins are often used as definitive signatures of a stressed condition for an organism (Heckathorn et al. 1999)

from Red Point, Caneel, and Cruz Bay (Fig 7; $p < 0.001$; ANOVA, Holm–Sidak post hoc test). GRP75 levels in the Trunk Bay, Tektite, and Hawksnest populations were not significantly different from each other, but were significantly higher than Red Point, Caneel and Cruz Bay populations. Heat-shock protein 60 (Hsp60) levels between Red Point and Caneel populations were not significantly different (Fig. 7; $p < 0.001$; ANOVA, Holm–Sidak post hoc test). Trunk Bay and Hawksnest were significantly higher than Red Point and Caneel, though Hawksnest was significantly different from Tektite (Fig. 7; $p < 0.001$; ANOVA, Holm–Sidak post hoc test). Cruz Bay was significantly higher than the other five populations, and almost 2.5 times higher than Red Point/Caneel (Fig. 7; $p < 0.001$; ANOVA, Holm–Sidak post hoc test). Ubiquitin levels were not significantly different among Red Point, Caneel, and Tektite,

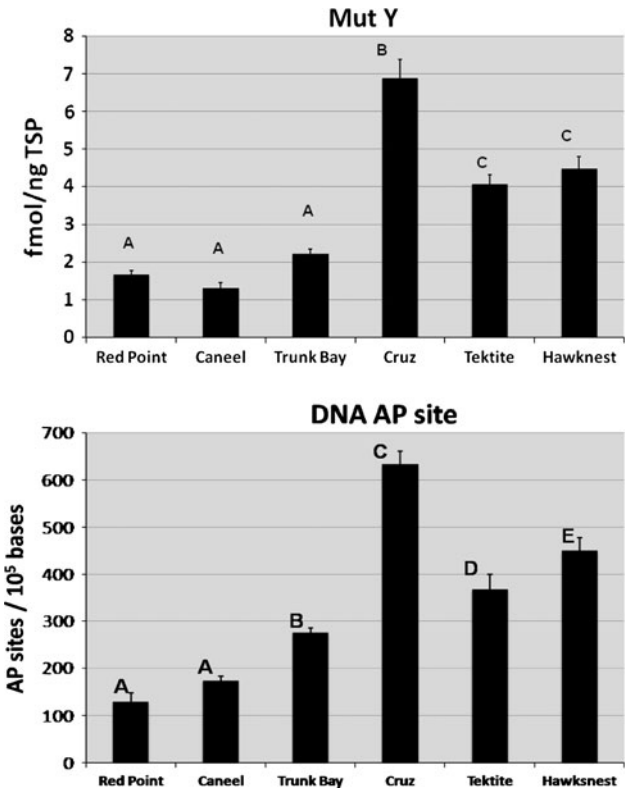


Fig. 4 Genomic integrity markers. *Bars* show untransformed means (±SE), n = six corals per site. Treatment means with different *uppercase letters* differed slightly at $\alpha = 0.05$ using the two different post hoc tests described in "Materials and methods" and "Results" sections

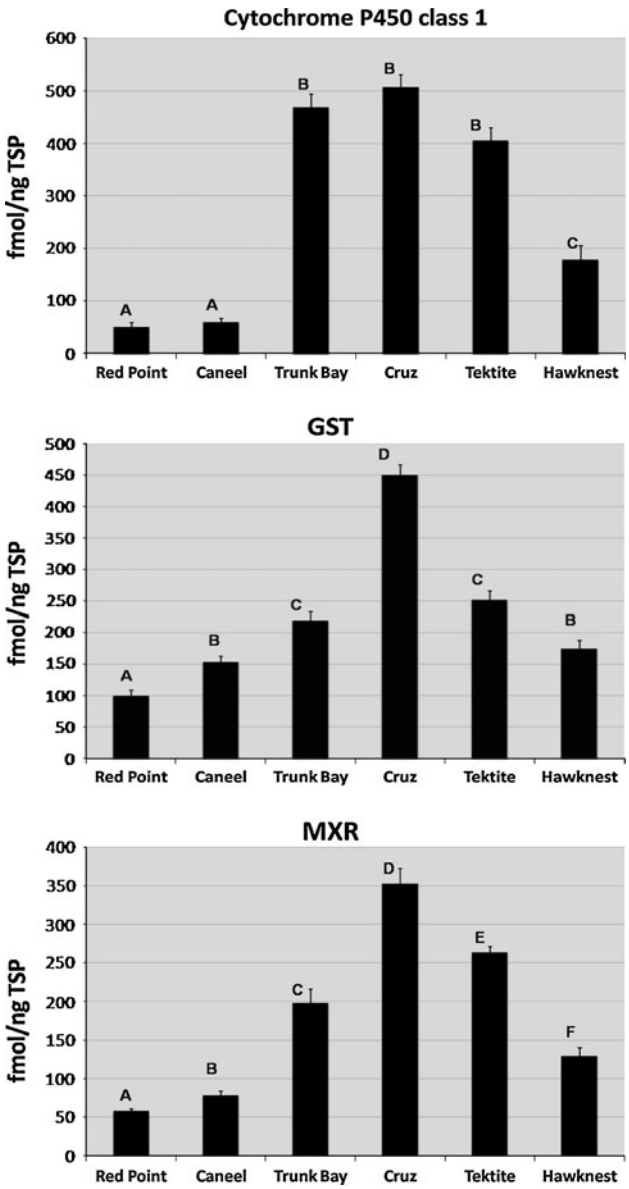


Fig. 5 Xenobiotic response markers. *Bars* show untransformed means (±SE), n = six corals per site. Treatment means with different *uppercase letters* differed slightly at $\alpha = 0.05$ using the two different post hoc tests described in "Materials and methods" and "Results" sections

while both Trunk and Hawksnest population had significantly higher accumulation than the other four populations (Fig. 7; $p < 0.001$; ANOVA, Holm–Sidak post hoc test). Cruz Bay had significantly lower levels of ubiquitin compared to the other five populations (Fig. 7; $p < 0.001$; ANOVA, Holm–Sidak post hoc test). Small heat-shock proteins (sHsp) were not significantly different among the populations of Red Point, Caneel, Trunk Bay, and Tektite (Fig. 7; $p < 0.001$; Kruskal–Wallis ANOVA, Student–Newman–Keuls post hoc test). Cruz Bay and Hawksnest had significantly elevated levels of sHsps (Fig. 7; $p < 0.001$; ANOVA, Holm–Sidak post hoc test).

Microbiology

Microbiology was conducted to determine whether these reefs were experiencing (a) elevated bacterial input (total bacterial counts); if so, are they from (b) fecal input (TC, FC and *E. coli*) and c) human or wildlife sources (*E.coli* and FC).

Testing to determine optimal sampling time in relation to tidal cycle indicated that *E. coli* had the highest counts 1 h after high tide whereas total coliform counts were more consistent throughout the tidal cycle only reaching a low 1 h before high tide (Fig. 8). All subsequent sampling occurred 1 h after high tide. Total coliform counts for Cruz Bay, Caneel Bay and Trunk Bay were 134, 55 and 78 cfu/100 ml, respectively. *E. coli* counts for Cruz Bay, Caneel Bay and Trunk Bay were 102, 13, and 46 cfu/100 ml while FC counts for Caneel and Trunk were 4 and 28 cfu/100 ml (Fig. 9). Fecal coliforms counts for Cruz Bay were not determined at this spring timepoint, though collections 3 months earlier had fecal coliform levels of 96 cfu/ml. Total bacterial counts for Cruz, Caneel and Trunk were

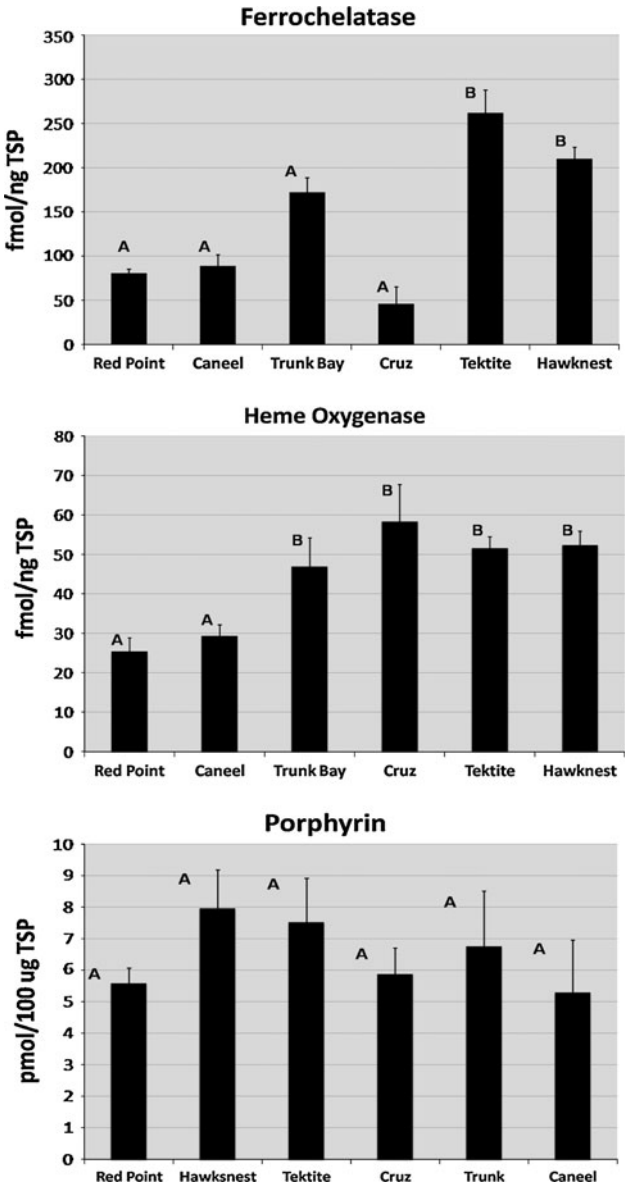


Fig. 6 Porphyrin metabolism markers. *Bars* show untransformed means (±SE), n = six corals per site. Treatment means with different *uppercase letters* differed slightly at $\alpha = 0.05$ using the two different post hoc tests described in "Materials and methods" and "Results" sections

1.73×10^6, 4.88×10^5 and 5.33×10^5 bacterial per ml seawater (Fig. 9).

Contaminant analysis

None of the target analytes (Table 2) were detected in surface sediments at Red Point, Caneel, or Trunk Bay (Table 3). At Cruz Bay, a number of organochlorine and nitrogen:phosphorous analytes were detected, though no semi-volatile polyaromatic hydrocarbons were detected (Table 3). At both Tektite and Hawksnest, only polyaromatic hydrocarbons were detected in surface sediment samples (Table 3).

Discussion

The first steps for an investigating a declining natural resource are to (1) characterize the physiological condition of the target population(s) and (2) document putative stressors (Boehm et al. 1995a, b; Downs et al. 2006a, b). An actual reference population or a justified "idealized" nominal range for the parameter values expected in a healthy population also needs to be established before any interpretation of a health differential can be stated (Downs 2005). The coral population at Red Point was used as a reference-comparison to the five other sites in this study because it (1) lacks any significant anthropogenic activity within its watershed, (2) lacks significant visitation by small water craft, divers and snorkelers, (3) lacks significant advection deposition because it is on the dominant windward side of the island, (4) exhibited no signs of recent coral mortality (~past 4 years), (5) showed an abundance of coral recruits, and (6) had cellular-stress marker patterns which correlated with *P. astreoides* stress marker patterns from Sapodilla Cayes, a marine protected area more than 30 miles off the coast of southern Belize during the same season (Fig. 3a) (Boehm et al. 1995b). Hence, Red Point was used as a reference to determine if there are meaningful differences in cellular diagnostic marker accumulations in the other five coral populations. It should also be noted that sampling occurred during the 14th week of a 15 week drought period, which would have an effect on the accumulation of contaminants from run-off.

Caneel

Caneel Bay resides within the Virgin Islands National Park at St. John and is surrounded by Caneel Bay Resort. The 170-acre resort contains manicured lawns, a back-up power generator, a desalination plant, and a dock for watercraft no longer than 100 ft. Along 230 m of the south coast of Caneel Bay, there is an extensive coral community that resides within 15 m of the shore line (Fig. 2b), consisting of *Diploria* spp., *Montastrea* spp., *Porites* spp., *Millepora* spp., *Mycetophyllia* spp., and *Acropora palmata*. Obvious potential impacts to corals would be run-off of pollutants from resort activities, pollution from moored boats, and fresh-water and sediment run-off resulting from severe storms. The only cellular markers that were significantly elevated in Caneel compared to Red Point were MXR and GST, indicating that this population was responding to an organic xenobiotic. No other markers were elevated,

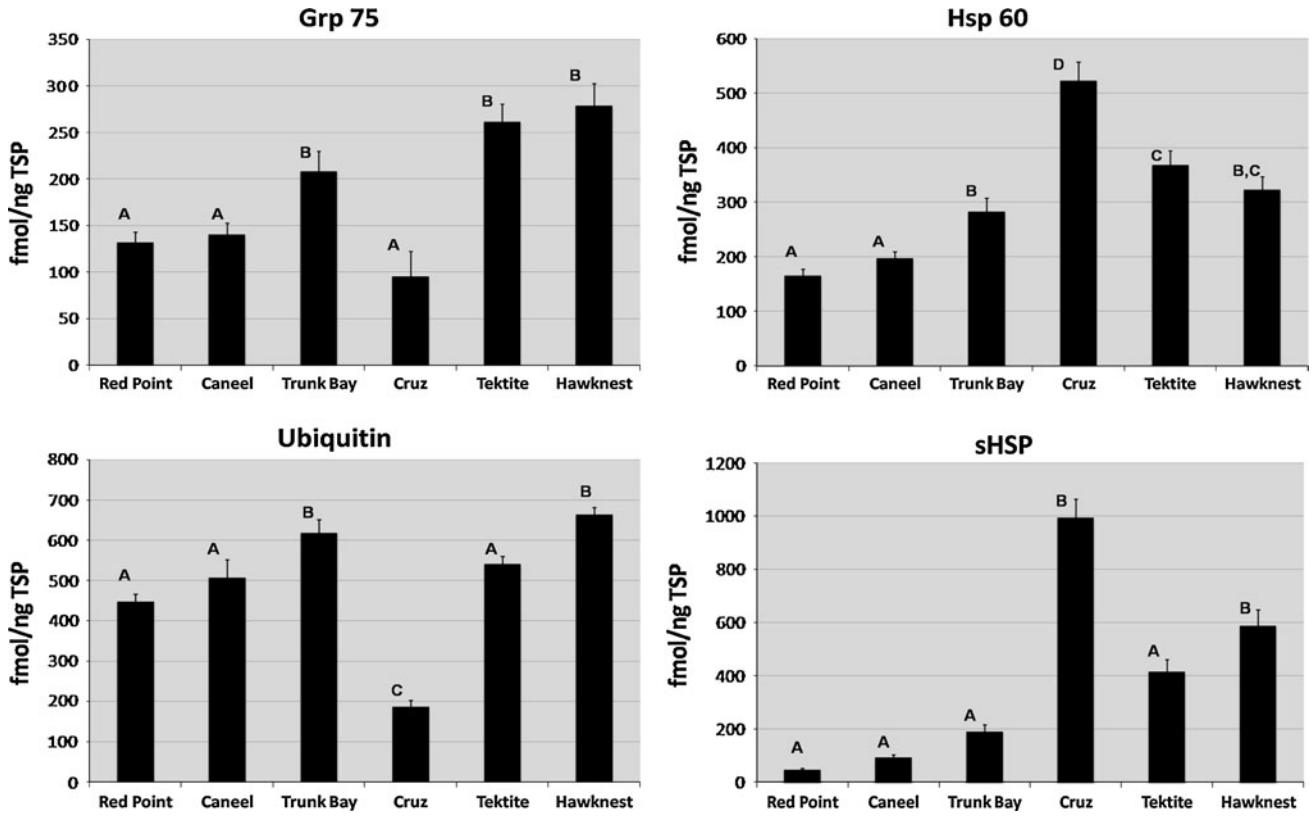


Fig. 7 Protein chaperoning and degradation markers. *Bars* show untransformed means (±SE), n = six corals per site. Treatment means with different *uppercase letters* differed slightly at $\alpha = 0.05$ using the two different post hoc tests described in "Materials and methods" and "Results" sections

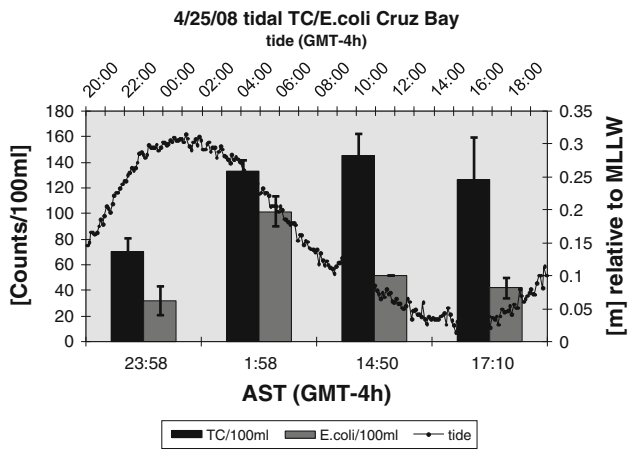


Fig. 8 Relationship between tidal stage and bacterial counts recovered. Total coliform (TC/100 ml) and *Escherichia coli* (*E.coli*/100 ml) were sampled at Cruz Bay to determine optimal collection times. *Bars* show untransformed means (±SE), n = three water samples per site

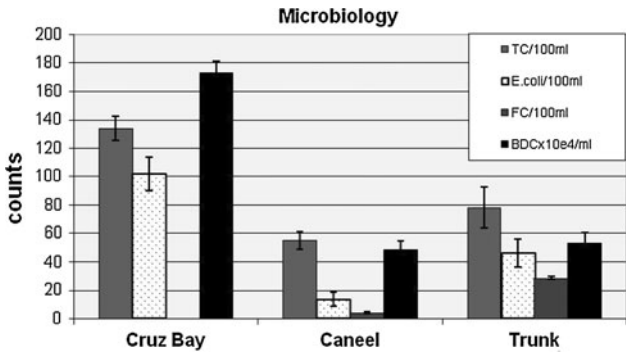


Fig. 9 Environmental microbiology. Total coliform (TC/100 ml), fecal coliform (FC/100 ml), *Escherichia coli* (*E.coli*/100 ml) and (microscopic) bacterial direct counts (BDC × 10^4/ml) for three different sampling sites (Cruz Bay, Caneel Bay and Trunk Bay), *Bars* show untransformed means (±SE), n = two water samples per site

suggesting that though Caneel may be responding to low level xenobiotics, there was no indication of a stressed or pathological physiological condition. Levels of TC, *E. coli* and FC were overall low (5:13:4 cfu/100 ml) suggesting that the coliform counts are likely due to sources other than a direct sewage source (e.g., terrestrial run-off and soil microbes). Fecal coliform levels were below action levels set by US EPA, USVI, and EU standards, suggesting that there was little exposure to sewage or other fecal matter from the Caneel watershed. Lack of detection of any of the contaminant target analytes in marine surface sediment both

Table 2 List of target analytes for analysis using a gas chromatograph-mass spectrometer detector

Nitrogen/phosphorus	Organochlorine	Semi-volatiles
Ametryn	Alachlor	Acenaphthylene
Atraton	Aldrin	Anthracene
Atrazine	Chlordane-α,γ	Benzo[a]anthracene
Bromacil	Chlorobenzilate	Benzo[b]fluoranthene
Butachlor	Chloroneb	Benzo[k]fluoranthene
Butylate	Chlorothalonil	Benzo[ghi]perylene
Carboxin	Chlorpyrifos	Benzo[a]pyrene
Chlorpropham	DCPA	2-Chlorobiphenyl
Cycloate	4,4′-DDD	Chrysene
Cyanazine	4,4′-DDE	Dibenz[a,h]anthracene
Diazinon	4,4′-DDT	2,3-Dichlorobiphenyl
Dichlorvos	Dieldrin	Bis(2-ethylhexyl)adipate
Diphenamid	Endosulfan I,II, sulfate	2,4-Dinitrotoluene
Disulfoton	Endrin	2,6-Dinitrotoluene
EPTC	Endrin aldehyde	Fluorene
Ethoprop	Etridiazole	Hexachlorobenzene
Fenamiphos	Hexachlorocyclohexane–alpha, beta, delta, gamma	2,2′,4,4′,5,6′-Hexachlorobiphenyl
Fenarimol	Heptachlor	2,2′,3,3′,4,4′,6-Heptachlorobiphenyl
Fluridone	Heptachlor epoxide (isomer B)	Hexachlorocyclopentadiene
Hexazinone	Methoxychlor	Indeno[1,2,3-cd]pyrene
Merphos	*Cis*-permethrin	Isophorone
Methyl paraoxon	*Trans*-permethrin	2,2′,3,3′,4,5′,6,6′-Octachlorobiphenyl
Metolachlor	Propachlor	2,2′,3′,4,6-Pentachlorobiphenyl
Mevinphos	*Trans*-nonachlor	Phenanthrene
MGK-264	Trifluralin	Pyrene
Molinate		2,2′,4,4′-Tetrachlorobiphenyl
Napropamide		2,4,5-Trichlorobiphenyl
Norflurazon		Pentachlorophenol
Pebulate		
Prebane (terbutryn)		
Prometryn		
Pronamide		
Propazine		
Simetryn		
Simazine		
Tebuthiuron		
Terbacil		
Terbufos		
Tetrachlorvinphos (stirofos)		
Triadimefon		
Tricyclazole		
Vernolate		

near the dock and in the coral community suggests that whatever xenobiotic(s) the corals are responding to were not among those screened in our analysis. Sunscreen compounds, such as oxybenzophenones were not detected in the water column at this site (data not shown). Other commonly occurring contaminants in Caribbean waters are cuprous oxide, tributyl tin, and other booster biocides in boat antifoulant paint, which were not screened, but the close proximity of moored boats warrants further investigation (Carbery et al. 2006; Konstantinou and Albanis 2004).

Table 3 List of target analytes and their concentration in marine surface sediment samples extracted using an accelerated solvent extraction method and analyzed using a gas chromatography system with a mass spectrometer detector

	Red Point	Caneel	Trunk	Cruz	Tektite	Hawksnest
Sample 1	Nothing detected	Nothing detected	Nothing detected	Nothing detected	Nothing detected	Nothing detected
Sample 2	Nothing detected	Nothing detected	Nothing detected	Nothing detected	50 ppb phenanthrene 8 ppb anthracene 120 ppb pyrene 44 ppb benzo[a]anthracene 38 ppb chrysene 14 ppb benzo[a]fluoranthene 3 ppb benzo[k]fluoranthene 20 ppb benzo[a]pyrene	Nothing detected
Sample 3	Nothing detected	Nothing detected	Nothing detected	43 ppb DDE 2 ppb DDD 2 ppb α-chlordane BCC pyrene BBC γ-chlordane BBC trans-Nonachlor BBC 2,2,4,4,5,6-hexachlorobiphenyl BBC chlorobenzilate BBC prometryn BBC terbutryn BBC metochlor	12 ppb phenanthrene 2 ppb anthracene 24 ppb pyrene 10 ppb benzo[a]anthracene 9 ppb chrysene 3 ppb benzo[b] fluoranthene 1 ppb benzo[k]fluoranthene 4 ppb benzo[a]pyrene	2 ppb phenanthrene 4 ppb pyrene 2 ppb benzo[a]anthracene 3 ppb chrysene 2 ppb benzo[b]fluoranthene 1 ppb benzo[a]pyrene BBC benzo[k]fluoranthene

Sample number corresponds with exactly where samples were collected, indicated in Fig. 2

Hawksnest

Hawksnest Bay resides within the Virgin Islands National Park at St. John, famous for its beach and was once a large nesting beach for Hawksbill turtles. The slopes of Hawksnest Bay watershed contain a small residential community, cottages of Caneel Bay Resort, a large cistern for Caneel Bay Resort, an asphalt dump, a lumberyard, and a community health center. Along the Hawksnest coastline, there is a parking lot with picnic facilities, and pit toilets as part of the National Park Service visitor facilities. Corals at Hawksnest exhibited elevated DNA AP lesions and MutY, indicating that this population was exposed to a genotoxic stressor. All three xenobiotic markers were elevated, indicating that the corals were being exposed and responding to a hydrocarbon-based xenobiotic. Both porphyrin enzymes were elevated. Ferrochelatase may have been elevated in response to greater demand for cytochrome production for CYP P450 and electron-transfer reactions, while heme oxygenase may have been elevated because of CYP P450 suicide reactions or a higher demand for bilirubin, which is a lipophillic anti-oxidant (Fucci et al. 1983; Stocker and Ames 1987; Thunell 2000). Porphyria markers were not significantly elevated, indicating that the hydrocarbon xenobiotic did not induce an inhibition of porphyrin anabolism. All four markers of protein chaperoning and degradation were significantly elevated, indicating that corals were experiencing a stressed condition that could be pathological (elevation of sHsps), signifying that there may be a reduction in reproductive output, growth, and even immunity (Heckathorn et al. 1999; Feder and Hofmann 1999). Polyaromatic hydrocarbons (PAHs) were the only environmental contaminants detected by our analysis, indicating that these may be the xenobiotics to which the corals are responding. Concentrations of PAHs were detected only in sample #3, which was collected 8 m from shore immediately in front of the beach entrance of the visitor's pavilion, next to one of the sampled coral colonies. Possible sources for these PAHs are the barbeque grills or road run-off from the asphalt road. The cellular-stress marker pattern at Hawksnest is consistent with an exposure to PAHs with the exception of a lack of elevated porphryin levels. To resolve this, coral samples should be assayed for benzo[a]pyrene diol epoxide

adducted to either DNA or protein (as in Downs et al. 2002, 2006b).

Trunk

Trunk Bay also resides within the Virgin Islands National Park at St. John and is considered one of the most frequented spots by tourists and locals on St. John. On some occasions before 2004, densities of visitors on the beach could reach as high as 1,000 people or more (Rafe Boulon, U.S. NPS statistics). There are less than a half-dozen residences on the slopes in the Trunk Bay Watershed, and a four-acre National Park Service tourist compound with showers and lavatories. The coral population at Trunk Bay had significantly elevated levels of DNA damage lesions, but no elevation of MutY, suggesting the presence of a mutagen at this site, though repair of this oxidation lesion may be addressed by different glycosylases than the one assayed. All three xenobiotic response markers were significantly elevated in Trunk Bay compared to Red Point, indicating that these corals were responding to an organic xenobiotic, most likely a polyaromatic hydrocarbon-based compound. Heme oxygenase was the only porphyrin metabolism marker that was significantly elevated, suggesting that either hemin was becoming damaged or there is need for the antioxidant, bilirubin (Guengerich 2004; Marks 1985; Stocker and Ames 1987). Significant elevation of the two mitochondrial chaperonins, GRP75 and Hsp60, indicate that there is a shift in mitochondrial protein metabolism. Significant elevation of ubiquitin confirms that at least in the cytosol, there is an increase in protein degradation metabolism. The lack of significant accumulation of the small Hsps indicates that the corals' condition, though stressed, has not reached the point of being dire, i.e., meaning damage to proteins.

Total coliforms are a group of bacteria that are widespread in nature. All members of the total coliform group can occur in the gut of humans and animals, but can also originate from soil and submerged wood. Fecal coliforms, are a subset of total coliform bacteria with a more fecal-specific origin, though they can include bacteria (*e.g.*, *Klebsiella* associated with textile and paper mill waste) that are not necessarily fecal in origin. *Escherichia coli* are fecal coliform bacteria that are specific to fecal material from humans and other animals (US EPA 2002; http://www.epa.gov/volunteer/stream/vms511.html). These microbial indicators point to a fecal input into Trunk Bay. Fecal source tracking was not done in this study, so it could not be discerned if fecal matter comes from residence, swimmers, the shower/lavatories at the visitor center, from feral mammals, such as donkeys and ferrets, or the local seabird population. Trunk Bay is however classified as Class A waters and USVI water quality bacteria standards state that "existing natural conditions shall not be changed" (WATER QUALITY ASSESSMENT for the US Virgin Islands 2001). Since *E. coli* and fecal coliform levels at Caneel (13 cfu/100 ml and 4 cfu/100 ml, respectively) are substantially lower than those found at Trunk, the data suggests the possibility of input above natural conditions and possibly exceeding water quality standards for Class A waters. None of the environmental contaminants in Table 1 were detected, though levels of oxybenzophenones at this site between 90 parts per billion and 1 part per million were detected in the water column (Downs, unpublished data), raising the possibility that the cellular stress responses in corals at this site could be the result of benzophenone exposure.

Cruz Bay

Cruz Bay is predominantly outside of the National Park boundaries, with the northern shore of the embayment within Park jurisdiction. Cruz Bay has the highest population density on St. John, containing hundreds of residences, businesses, and has a high density of moored personal watercraft as well as docks for ferries. Corals in Cruz Bay had the highest levels of DNA damage and DNA repair markers compared to the other five sites, indicating a strong presence of a genotoxic stressor. Cruz Bay also had the highest accumulation of xenobiotic response markers, indicating the presence of a hydrocarbon xenobiotic. Cruz Bay showed a perplexing diagnostic marker pattern for porphryin metabolism. Ferrochelatase levels, though not statistically different from Red Point, were the lowest concentrations out of the six populations, indicating perhaps a bottleneck in heme production. Heme oxygenase levels were also the highest concentration out of the six populations, though again, porphyrin levels were not significantly different from Red Point. Protein chaperoning and degradation diagnostic markers also showed an unusual pattern. GRP75 levels were not significantly elevated, but Hsp60 levels were, suggesting that at least within the mitochondria, there was significant renaturation of denatured proteins, but not a significant increase in protein import. Ubiquitin levels were depressed, which by itself would suggest a major shift in the protein metabolic equilibrium. In conjunction with extremely high levels of sHsp expression, this suggests either a mis-regulation of ubiquitin expression or that protein damage is not occurring in the cytosol, but in organelles that are regulated by autophagy.

Winter fecal coliform counts (96 cfu/100 ml) indicated that Cruz Bay exceeded Class B water quality standards (70 cfu/100 ml) during this period. Total bacterial loads were twice as high as those in Caneel and Trunk Bay (direct counts). High bacterial loads, coupled with the comparatively high levels of fecal coliforms and *E. coli,* strongly suggests that this site is impacted by human fecal waste

water, most likely from grey-water discharges from personal watercraft moored in the bay. Though no PAHs were detected, a long list of pesticides, herbicides, and fungicides were detected, none of which are associated with booster anti-foulants, but are associated with treated lumber, pest control, and weed control. The breakdown products of DDT (dichlorodiphenyltrichloroethane) are DDD and DDE. Sediment samples were collected at the end of a three-month drought. Since there had been no new run-off into the Bay during the drought, this indicates that the source of DDT is not marine deposition, though atmospheric or run-off deposition cannot be excluded. The organochlorine compounds that were detected have all been banned by the U.S. EPA for at least the past 15-30 years, so their presence in the surface sediment is an enigma, especially the PCB species.

Tektite

Tektite Reef lies within Beehive Bay, a small cove on the southeastern tip of Great Lameshur Bay. Tektite has two small environmental mooring buoys for recreational day use at the shallow, eastern end of the reef (Fig. 2e). Because of the precipitous slope of the watershed ridge and relatively little depth of the drainage basin adjacent to Tektite, there is almost no access to this site by land—access to this site is by watercraft. Visitation to this site is intense, though only two crafts at a time can tie onto the buoys. Long-term monitoring of this site has been conducted by the U.S. National Park service and others, documenting that between 1987 and 1998, coral cover at this site increased 34% (Edmunds 2002). In 2005–2006, coral cover at Tektite declined by 48% in only 6 months and 54% loss in 10 months as a result of coral bleaching and a probable "post-infection" event (Norberg-King et al. 2005). The only environmental organic contaminants detected at the Tektite site were PAHs; the highest concentrations immediately within an 3 m radius of the mooring anchor, while lower concentrations were detected 30 m away from the buoy anchor, within the main structure of Tektite Reef. An exposure to PAHs at these concentrations would elicit a genotoxic response, a xenobiotic defense response, a shift in porphyrin metabolism, and a shift in protein chaperoning and degradation—all of which occurred in corals sampled at this site, with the exception of increased porphyrin accumulation. To confirm that corals are being exposed to PAHs and whether these PAHs are adversely impacting the health of corals, PAH-contaminant chemistry analysis of PAHs in coral tissues and assaying for benzo[a]pyrene diol epoxide adducted to DNA or protein should be conducted (Downs et al. 2002, 2006b).

For the resource manager, the detection of damage should set into motion a process that not only documents damage, but also establishes causation. This requires demonstrating mechanisms of toxicity for suspected causative agents and providing a weight-of-evidence argument that documents the exposure of the resource and associated injury (Boehm et al. 1995a; Cormier and Suter 2008; Cormier 2006; Downs et al. 2005a, b, 2006a). This study serves to illustrate the initial steps necessary to conduct an integrated environmental investigation into natural resource damage—coral reef degradation. We have provided significant evidence that corals within specific embayments of St. John Island are being exposed to different pollutants and that the physiological condition of these corals at some of these sites are stressed. Because the stress and contaminant profiles are different at each of the impacted sites, each site needs to be investigated as an independent case, not grouped together as a broad-regional phenomenon of coral stress and declines. Though our results have not provided definitive causal linkages, it has provided significant evidence for the type of agent affecting each of these sites and prescribes focusing on specific candidate-causative agents. For example, metal analysis should be included in future site investigations to account for copper leachate from residential treated lumber, cadmium from tires, and cuprous oxide and tributyl-tin species from boat anti-foulant paints. The least expensive and most meaningful 'next-step' to investigate these sites is to conduct pore-water toxicity and Toxicity Identification Evaluations in order to corroborate field-collected data (Carr and Nipper 2003; Norberg-King et al. 2005). As additional pieces of evidence are assembled, we expect to be able to identify causal links, determine the relative contribution of different stressors, and provide reasonable guidance for better informed management of the reefs in St. John, USVI.

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