Final Report: Southeast Florida Coral Biomarker Local Action Study

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Abstract

We tested the feasibility of using cellular diagnostics to link land-based sources of pollution with coral reef degradation. We collected tissue samples of mustard hill coral (*Porites astreiodes*) from four paired inshore and offshore stations off Broward Country, FL: 1) control sites (FTL1 and FTL 3) monitored by Broward County Department of Environmental Protection, 2) a sewage outfall (Hollywood Outfall: HWO2 and HWO3), 3) an inlet mouth (Port Everglades: PE2 and PE3), and 4) a sewage outfall adjacent to an inlet mouth (Hillsborough Inlet: HI2 and HI3). Coral cover was <4% at all sites and cluster analysis grouped all inshore sites (except HI) together based on having the least bare substrate and the most *Lyngbya*. Rates of sampling lesion regeneration were significantly greater than zero only at three inshore sites and the offshore biomonitoring control site. Cellular diagnostics revealed that corals at all sites were stressed compared to colonies from a more pristine site in the Bahamas. Offshore corals consistently had higher accumulations of stress markers than inshore colonies. Stress responses of corals from HWO and the Florida Keys National Marine Sanctuary were consistent with sewage exposure, while responses of offshore colonies were consistent with xenobiotic detoxification. Accumulations of biomarkers of xenobiotic response and decreased protein turnover were associated with decreased rates of coral regeneration. We compared these results to experimental corals exposed to water collected from Port Everglades. Differences between coral clones within a depth zone remained apparent even after two months' acclimation to lab conditions, suggesting a genetic component to coral responses. Exposure to Port Everglades water decreased accumulation of multidrug resistance protein compared to colonies in the artificial sea water control; the levels were similar to the response observed in the field. This project

is a first step in identifying the chain of causality between land-based pollutants, responses of individual reef-building corals, and health of coral reef communities in the South Florida watershed.

Keywords: biomarkers, coral, *Porites astreoides*, reefs, regeneration, stressor

Introduction

Coral reefs are declining world-wide due to deteriorating environmental conditions (Dustan and Halas 1987; Bryant and Burke 1998; Dustan 1999; Hoegh-Guldberg 1999; Wilkinson 1999; Gardner et al. 2003). Surveys indicate that 58–70% of coral reefs worldwide are threatened by anthropogenic activities (Bryant and Burke 1998; Wilkinson 1999; Hoegh-Guldberg 1999; Goreau et al. 2000; Gardner et al. 2003). Coral colonies that experience persistent environmental disturbance suffer mass mortality, reduced growth and reproductive rates, and increased prevalence and severity of disease (Richmond 1993; Hoegh-Guldberg 1999; Nystrom et al. 2000; Knowlton 2001; Porter and Tougas 2001; Patterson et al. 2002). Unfortunately, most coral monitoring and mapping programs cannot identify the causes of coral declines because these programs were designed to quantify baseline conditions and determine trends, not identify stressors (Risk 1999; Pennisi 2002; Vasseur and Cossu-Leguille 2003).

In 2003, the Southeast Florida Coral Reef Initiative Team (SEFCRI Team) identified land-based sources of pollution as a potentially critical factor influencing the long-term fate of coral reefs off southeastern Florida. Here, we report results of a project that integrates traditional coral monitoring techniques with a novel biotechnology – cellular diagnostics – to address how land-based sources of pollution identified by the SEFCRI Team affect coral reefs. Cellular diagnostics is a new approach to environmental health assessment. It is based on concepts and methodologies widely used in medical diagnostics and epidemiology, and combines biomarkers of exposure, physiological effect, and potential risk to identify likely environmental stressors and forecast their biological effects (Downs 2005). Cellular diagnostics was designed to

assess the cellular-physiological condition of reef-building corals and identify mechanisms of coral pathologies (Downs et al. 2000, 2002; Brown et al. 2002; Woodley et al. 2002; Fauth et al. 2003; Downs 2005). Parameters evaluated include cell membrane integrity and composition, anti-oxidant redox status, protein metabolic condition, xenobiotic detoxification pathways, intra-cellular metal regulation, and genomic integrity. Cellular diagnostics works because environmental stressors affect organisms by overwhelming defenses at lower levels of the biological hierarchy: molecular, cellular, and organismal-level homeostatic processes. Stressors then reduce individual fitness, which cascades into altering demographic parameters, and diminishes the structure, function, and resilience of ecological communities. By evaluating coral responses at the molecular, cellular, organismal, and community levels, scientists can provide resource managers with critical information needed to identify and ameliorate stressors before an ecosystem-scale environmental crisis occurs (Fauth et al. 2003; Downs et al. in press).

Most studies of environmental stress and response are limited to using a weight-of-evidence or ecoepidemiological approach to infer causality (Adams 2003). To strengthen inferences of our field study, we conducted a laboratory experiment that exposed coral colonies to water collected from a putative source of stress. This mechanistic approach permits rigorous application of the scientific method, including the potential to falsify hypotheses derived from field sampling. By explicitly testing the chain of causality, we provide the evidence that resource managers, citizens, and legislators need to evaluate when making critical decisions about the effects of sewage

discharges, shipping channels, and other land-based sources of pollution on southeast Florida coral reefs.

Materials and methods

Study sites and species

The high-latitude coral reefs of southeastern Florida off Broward County are highly variable communities comprised of typical Caribbean taxa. These reef communities occupy four parallel submarine ridges. The two middle ridges (approx. 10 and 20 m depth) are remnants of earlier reefs that were drowned as sea level rose after the last glaciation (Moyer et al. 2003). Benthic cover is dominated by sponges, macroalgae and soft corals, and hermatypic or stony coral coverage comprise just 1-6% of projected cover (Moyer et al. 2003, this study). Biogeographic patterns on southeastern Florida reefs do not mirror those of reefs further south in the Florida Keys or the Caribbean basin. Species richness of southeastern Florida reefs tends to increase from north to south while diversity and evenness do not. This has been attributed to the harsher climate associated with higher latitude, including reduced winter water temperatures, higher frequency of major storms, and disturbances associated with close proximity to the Miami-Ft. Lauderdale-West Palm Beach metropolitan region.

Our sampling scheme was designed for analysis of variance; we sampled coral reefs near a sewage outfall, an inlet mouth, a sewage outfall located within an inlet mouth, and a biomonitoring control site off Broward Country, Florida, USA (Table 1, Fig. 1). Our focal species was the mustard hill coral (*Porites astreoides*: Fig. 2), which is a common reef-building coral throughout the western Atlantic, Gulf of Mexico, and the

Table 1. Paired sampling stations and nearby potential sources of land-based pollution off the southeastern Florida coastline, Broward County, Florida, USA.

Station/ depth	Ridge position	Latitude/Longitude	Location	Potential source of land-based pollution
FTL1 9-9.5 m	1st	26.095968 -80.052802	Fort Lauderdale	Biomonitoring control site
FTL3 17.5 m	3rd	26.095183 -80.046402	Fort Lauderdale	Biomonitoring control site
HWO2a 9-9.5 m	2nd	26.011414 -80.059973	Hollywood	Sewage outfall
HWO3 15.5-	3rd	26.011554 -80.052497	Hollywood	Sewage outfall
PE2 8 m	2nd	26.054585 -80.057387	Port Everglades	Shipping channel
PE3 14-15.5	3rd	26.059902 -80.050184	Port Everglades	Shipping channel
HI2 9-9.5 m	2nd	26.142301 -80.047515	Hillsboro Inlet	Sewage outfall + Shipping channel
HI3 15.5-	3rd	26.151344 -80.039077	Hillsboro Inlet	Sewage outfall + Shipping channel

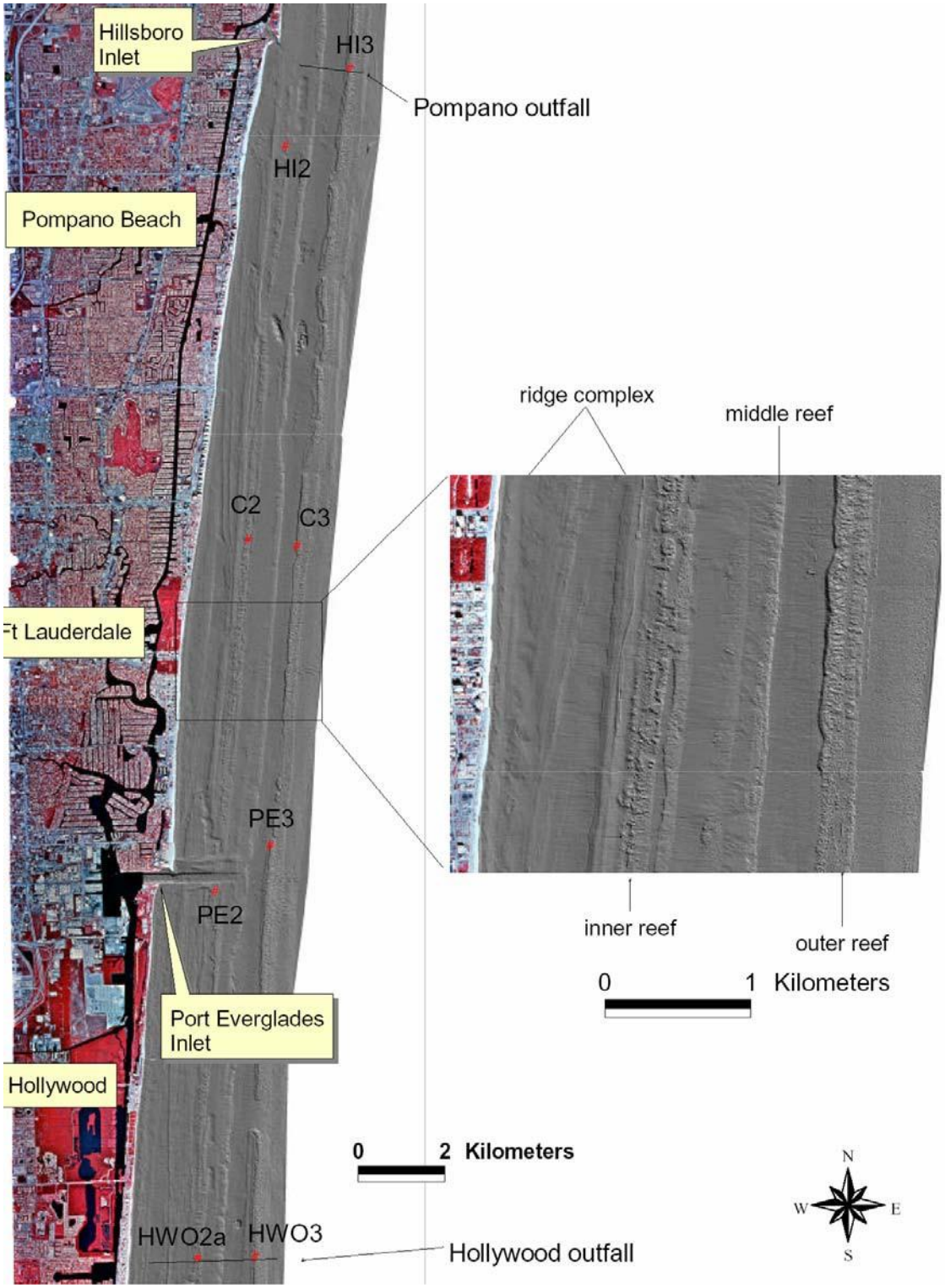

Figure 1. Chart of paired sampling locations off Broward County, Florida. Sites labeled C2 and C3 correspond to sites FTL1 and FTL3, respectively, in the text of this report.

Figure 2. A colony of mustard hill coral (Porites astreoides) with a sampling lesion filled with nontoxic modeling clay (gray oval). Note the recent tissue loss (whitish spots) on the opposite side of this colony. Ruler markings are in cm.Caribbean Sea. Porites astreoides is a hardy species that already was verified as acceptable for cellular-diagnostic analyses.

We used AGRRA (Atlantic and Gulf Reef Rapid Assessment) protocols to assess coral condition (Kramer et al. 2005). We selected five colonies of *Porites astreoides* at each site and measured their length, width, height, distance and direction from the U-post that marked our sites. We calculated colony surface area (SA) using the equation for a cone:

$SA = \Pi R \sqrt{(R^2/2 + h^2)}$

where R = (colony length + width)/2 and h = colony height. We also estimated percent old and recent tissue loss, recorded signs of bleaching, disease and overgrowth, and

recorded colony depth (m).

Ecosystem assessment

To assess coral reef community structure, we examined digital video imagery collected along a 15-25 m transect at each of the paired stations in January, 2005. Video was converted to single-frame images through frame grabbing and subsequent digital processing. Point counting (15 pts/frame) was used to estimate projected coverage (percent cover) of stony corals and other species (Dustan et al. 1999). We also estimated percent cover of functional groups including gorgonians, zooanthids, porifera, macroalgae, and members of the cyanobacteria genus *Lyngbya*.

To quantify environmental variation among sites, we deployed in submersible cases two HOBO H08 temperature loggers (Onset Corporation, Bourne, MA) that recorded water temperature hourly (accuracy: ± 0.7° at 21° C). We collected sediment by attaching a 5.1 cm diameter x 60 cm long trap to a steel U-post painted with epoxy. One sediment trap was deployed at each site in January and retrieved in August. We monitored salinity of surface water using using a VeeGee Model A366ATC refractometer, turbidity with an Orbeco-Hellige Model 966 turbidimeter (Orbeco Analytical Systems, Farmingdale, NY) and water temperature, dissolved oxygen, and conductivity using a Yellow Springs Instruments Model 556 Multiprobe System (Yellow Springs Instruments, Inc., Yellow Springs, OH) . We also collected ~250 mL bottom sediment sample and 4 L of surface water in U.S. Environmental Protection Agency- certified glass containers. These samples remain stored at -80° C at the University of Central Florida (UCF).

Organismal assessment: lesion regeneration

We used a new method to assess the health of coral colonies – their ability to heal sampling lesions (Fisher et al. in preparation). Briefly, when we removed coral tissue and its underlying skeletal structure for cellular-diagnostic analyses, a standardized, circular lesion was created, which was approximately 1-2 cm^2 in area, 3 mm deep, and surrounded by live tissue. We filled the hole with nontoxic modeling clay (Fig. 2) to prevent colonization by fouling and bioeroding organisms, then measured its major and minor chords using a ruler. We then photographed each lesion using a digital videocamera or digital camera. We included a ruler or scale bar to calibrate measurements. We re-measured and photographed each lesion in August, 2005 to observe lesion regeneration over time (Fisher et al. in preparation).

We used *in situ* measurements to calculate the area (A_L) and perimeter (P_L) of all lesions, which remained completely surrounded by live tissue (Type I lesions; Meesters et al. 1997). We calculated lesion area using the equation for an ellipse, $A_L = \pi ab$, where a and b are one-half of the length and width, respectively, of a lesion. We expressed regeneration rate as $mm^2\ d^{-1}$ for statistical analyses.

Cellular diagnostics

Reagents

Chemicals for buffered solutions were obtained from Sigma Chemicals Co. (St. Louis, Missouri, USA). PVDF membrane was obtained from Millipore Corp. (Bedford, Massachusetts, USA). Antibodies against all cellular parameters and their calibrant standards were obtained as gifts from Robert Richmond, University of Hawai‘i, which in

turn were gifts from EnVirtue Biotechnologies, Inc. (Winchester, Virginia, USA). Antibodies were raised against an 8–12 residue polypeptide conjugated to ova albumin. Antigens were designed based on extremely conserved and unique domains found within the target protein. Rabbits were immunized with the antigen with a Ribi-adjuvant carrier. All antibodies used in this study were immuno-purified with a Pierce SulfoLink Kit (cat.# 44895) using the original unconjugated peptide as the affinity binding agent. Anti-rabbit conjugated horseradish peroxidase antibodies were obtained from Jackson Immunoresearch (West Grove, PA, USA).

Sampling coral colonies

We collected samples (1.5 cm diameter) from five colonies at each site in January, 2005. Samples were biopsied using a leather punch and placed in labeled, opaque film canisters. On deck, under shaded conditions, water was quickly removed from the film canister, blotted dry on paper towels, placed in labeled containers, and transferred to dry ice. Samples were stored at -80° C until analysis.

Sample preparation, ELISA validation, and ELISA

Frozen coral samples were ground to a powder using a liquid nitrogen-chilled ceramic pestle and mortar. Samples (~10 mg) of frozen tissue were placed in 1.8 ml microcentrifuge tubes with 1400 µl of a denaturing buffer consisting of 2% SDS, 50 mM Tris–HCl (pH 6.8), 15 mM dithiothreitol, 10 mM EDTA, 0.001 mM sorbitol, 7% polyvinylpolypyrrolidone (wt/vol), 0.1% polyvinylpyrrolidone (wt/vol), 0.01 mM alpha-tocopherol, 0.005 mM salicylic acid, 2 mM benzamidine, 0.04 mM Bestatin, 0.001 E-64, 2 mM phenylmethylsulfonyl fluoride, 0.01 mM apoprotin, 5 :Ma-amino-caproic acid, and 1 :g/100 ul pepstatin A. Samples were heated to 92° C for 3 min, vortexed for 20 s, incubated at 92° C for another 3 min, and then incubated at 25° C for 5 min. Samples were

centrifuged at 10,000 · g for 10 min. Supernatant free of the lipid/glycoprotein mucilage matrix was transferred to a new tube, centrifuged at 10,000 · g for 5 min and free supernatant again was transferred to a new tube and subjected to a protein concentration assay (Ghosh et al. 1988).

To ensure equal sample loading, 20 µg of total soluble protein (TSP) of samples were loaded onto a 12.5% SDS-PAGE gel (16 cm), the gel was run until the bromophenol blue dye front was near the bottom, stained with a Coomassie blue solution (BB R-250) overnight, and then destained for 4 h with multiple washes of destaining solution. Equal loading was determined by visualization and optical density using a Canonscan scanner and analysis performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). This protocol visually validated the protein concentration assay and ensured that sample artifact did not occur between the time of sample homogenization and sample analysis.

One-dimensional SDS-PAGE and western blotting validated the legitimacy of an ELISA (enzyme linked immunosorbent assay) on this species of coral using a specific antibody (Downs 2005). Five to 15 :g TSP of coral supernatant was loaded onto a 20-, 16-, or 8-cm SDS-polyacrylamide gel with various concentrations of bis/acrylamide. A Tri(2-carboxyethyl) phosphine (TCEP) concentration of 1 mM was added to gels loaded with samples to be assayed with antibody to the chloroplast sHsp and the invertebrate sHsp. Tri(2-carboxyethyl)phosphine is a reductant that can be used in acrylamide gels without interfering with acrylamide polymerization. Proteins can often spontaneously form disulfide bonds in the loading buffer, in the stacker gel, and in the separating gel, causing homomeric or heterometic dimerization. Proteins (e.g., small heat-shock

proteins) that dimerize under adverse environmental conditions readily aggregate under standard SDS-PAGE procedures. Gels were blotted onto PVDF membrane using a wet transfer system. Membrane was blocked in 7% non-fat dry milk, and incubated with the primary antibody for 1 h. Blots were washed in tris- buffered saline (TBS)-0.05% Tween (v/v) four times, and incubated in a horseradish peroxidase-conjugated secondary antibody solution for 1 h. Blots were washed four times in TBS and developed using a chemiluminescent reporter system.

Once validated, antibodies and samples were optimized for ELISA using an 8 · 6 · 4 factorial design (Crowther 1999). Every ELISA assay must be optimized for proper concentration of protein loading, antibody titer, sample-to-standard calibration, and handling procedures as a measure of quality control and quality assurance (Downs 2005). A Bio-Tek 404 plate washer was used in conducting the ELISAs using a 96-well micro-titer plate format. Antibodies were developed using a luminol-based chemiluminenscent solution and documented using a Bio-Tek fluorescent/luminescent microplate reader.

Samples were assayed with Haereticus Environmental Laboratory's antibodies against heat shock protein 60 (Hsp 60), cytochrome P450-2 class (Cyp 2), cytochrome P450-6 class (Cyp 6), Glucose-regulated protein 75 (Grp 75), total small heat shock proteins (sHsp), ubiquitin, copper-zinc superoxide dismutase (Cu/Zn SOD), cnidarian glutathione-S-transferase (GST), ferrochelatase (FC) and multi-drug resistance protein (MDR). The biological significance of each cellular-diagnostic parameter (biomarker) is summarized in Table 2. Samples were assayed in triplicate with intra-specific variation < 6% for the whole plate. An eight-point calibrant curve using a calibrant relevant to each antibody was plated in triplicate for each plate.

Table 2 Cellular-diagnostic parameters and their biological significance

Parameter	Interpretation
Copper-Zinc superoxide dismutase (Cu/Zn SOD)	Superoxide dismutases are enzymes that catalyze superoxide radicals to molecular oxygen and hydrogen peroxide, and comprise a main antioxidant defense pathway (Wu et al. 1999). Increased SOD levels have been linked to increased longevity and tolerance to ischemic/reperfusion events and oxidative stress (Fridovich 1995).
Heat shock protein 60 (Hsp 60)	Heat shock proteins are molecular chaperones universal to all eukaryotic cells. During stress, Hsp's protect cells from elevated temperature and are important in repairing cellular damage (Near et al. 1990; Welch 1993). Hsp 60 accumulates in response to stress, specifically increased protein synthesis and denaturation.
Total small heat shock proteins (sHsp)	Cnidarians have up to 5–6 major sHsp isoforms, including αB-crystallin, Hsp22, Hsp23, Hsp26, and Hsp28 (Downs et al. 1999). In general, sHsp's are absent under optimal growing conditions and are only elicited by stress. For example, αB- crystallin is only found in the cytosol of animals, where it protects cytoskeletal elements during stress (Derham and Harding 1999). Thus, the presence and concentration of different small heat-shock proteins reflects the physiological status of several cellular metabolic and structural pathways.
Glucose-regulated protein (Grp)	Glucose regulated protein is a mitochondrial matrix protein related to the Hsp 70 family and is induced under conditions of low glucose and other environmental stresses. It also is involved in various chaperoning functions and possibly in antigen recognition, cell proliferation and senescence (Pockley 2001). Concentrations of Grp often are used as indicators of nutritional stress.
Glutathione-S-transferase (Gst)	Glutathione-S-transferase detoxifies genotoxic and cytotoxic xenobiotic electrophiles by conjugating them to glutathione (Ketterer et al. 1988). It also can repair DNA by detoxifying DNA hydroperoxides (Tan et al. 1988) and is a main defense for detoxifying 4-hydroxynonenal, an extremely reactive product of lipid peroxidation that cross-links proteins and forms adducts with DNA (de Zwart et al. 1999).

Parameter	Interpretation
Ubiquitin	Ubiquitin is a 76-residue protein found in most phyla and marks proteins for rapid degradation. Ubiquinated proteins are degraded by proteolytic enzymes known as proteosomes. Accumulations of ubiquitin indicate increased protein degradation and turnover. Thus, ubiquitin levels are an index of the structural integrity of the protein component of the cell superstructure.
Ferrochelatase (FC)	Ferrochelatase is an indicator of changes in metabolic state, usually in response to stress. Ferrochelatase inserts ferrous iron into protoporphyrin IX to form heme, which is essential for cellular metabolism and detoxification (Ferreira 1999). For example, cytochrome *c* requires a form of heme to become an active electron carrier and cytochrome P450 requires heme to function. In addition, breakdown of heme leads to formation of billirubin and biliverdin (Smith et al. 1994); the former is a very effective anti-oxidant, rivaling Vitamin E as a scavenger of lipid hydroperoxyls.
Multidrug resistance protein (MDR)	Multidrug resistance protein protects against toxicity of 4-hydroxynonenal, a major product of lipid peroxidation that can inhibit DNA, RNA, and protein synthesis, stop the cell cycle, disrupt mitochondrial functions, and produce pathological disorders (Renes et al. 2000). Elevated MDR accumulations indicate defenses were mobilized against cellular stress.
Cytochrome P450-2 class (CYP 2)	Cytochrome P450-2 class mainly detoxifies electrophilic carcinogens, drugs, and environmental pollutants.
Cytochrome P450-6 class (CYP 6)	Overexpression of CYP 6 has been implicated in the evolution of pesticide resistance – including to DDT – in arthropods and other invertebrates (Walters et al. 1992).

Laboratory stressor-challenge experiment

Coral collection and maintenance

Four colonies of *Porites astreoides* were obtained from reefs off Fort Lauderdale, Florida and transported to Nova Southeastern University Oceanographic Center (NSUOC). Two colonies were collected from a depth of 7 m and two colonies from 15 m. In the laboratory, colonies were placed into a 150 L holding tank linked to a ~520 L closed system equipped with a 3590 Lh^{-1} recirculation pump and an 80 L protein skimmer. Aquarium water consisted of a mixture (~1:1 ratio) of filtered natural seawater and reverse-osmosis/deionized water mixed with commercial sea salt. Additional water circulation within the holding tank was provided by submersible power heads. Tanks were illuminated by four 175 W, 10000 K metal halide bulbs on a 12 h photoperiod. Water temperature in the holding tank ranged from 23.8 - 28.3° C (25.8 ± 0.9; mean ± SD) and salinity between 35 - 36‰ (35.4 ± 0.4). Colony fragments acclimated in this tank for one month. Subsequently, twelve 13 mm diameter cores were removed from each colony using a diamond core drill. Cores were glued in an upright position to epoxy resin plugs using cyanoacrylate gel and allowed to recover in the holding tank for approximately 1 month. During this recovery period, coral tissue grew and extended down the side of each core.

Experimental manipulations

After the two-month acclimation period, coral cores were transferred to 24 separate, 9.5 L closed-system aquaria. Coral cores (two per tank) and experimental treatments were randomly assigned to each tank. Treatments consisted of a control exposed to filtered

natural seawater and reverse-osmosis/deionized water mixed with commercial sea salt as described previously, and a stressor challenge, which consisted of water collected daily at high tide from Port Everglades, filtered to 50 microns, and stored briefly in glass aquaria before use. Both the control and stressor treatments were replicated twelve times. During the experiment, water circulation and filtration were provided by Aquaclear Mini™ power filters and 50% water changes were performed every 12 h. Light conditions were similar to acclimation conditions. Temperature, salinity and pH of all tanks was tested every 24 hrs. Temperature was measured with Cole-Parmer Instrument Co. NIST-traceable mercury thermometer (accuracy 0.1°C) and averaged 25.7 ± 1.0°C. Salinity was determined with Atago ATC-S/Mill-E Refractometer and averaged 35.8 ±0.6 ppt. An Orion Research Model SA230 pH/mV/°C meter (electrode calibrated with NBS Standard buffers of pH 2, 7 & 9) $pH_{(NBS)}$ was used to calculate pH from millivolt measurements, which averaged 7.97 ± 0.73. Coral cores were maintained under experimental conditions for 4 days (96 h).

Ammonia, nitrite, nitrate, alkalinity and phosphate were measured in the water used for the water changes with LaMotte Scientific Test Kits. In the control tanks, ammonia averaged 0.13 ± 0.25 ppm; nitrite and nitrate remained below detection limits. Mean water alkalinity was 186 ± 3 ppm and mean phosphate concentration was 0.06 ± 0.03 ppm. In the treatment tanks with Port Everglades water, mean ammonia concentration was 0.16 ± 0.23 ppm; nitrite and nitrate remained below detection limits. Mean alkalinity was 192.0 ± 4.0 ppm and phosphate averaged 0.14 ± 0.08 ppm.

After each daily, 50% water change, ammonia, nitrite, nitrate, alkalinity, and phosphate were measured in two tanks (randomly selected control and treatment tank)

with LaMotte Scientific Test Kits. In both the control and treatment tanks, ammonia averaged 0.09 ±0.14 ppm; nitrite and nitrate remained below detection limits. In the control tanks, alkalinity averaged 189.0 ± 6.0 ppm and phosphate averaged 0.03 ± 0.03 ppm. In the treatment tanks, alkalinity averaged 194.0 ± 3.0 ppm and phosphate averaged 0.05 ± 0.0 ppm. One-way analysis of variance indicated no significant differences in temperature or salinity between acclimation, control, and treatment conditions ($p > 0.05$). In addition, differences in pH, alkalinity, ammonia, and phosphate between control and treatment tanks were not statistically significant ($p > 0.05$).

At the end of the experiment, we recorded the position of all tanks relative to the lab door and aisle. These data were used as covariates to account for potential two-dimensional gradients in the laboratory. All corals were removed from tanks, gently blotted with paper towel to remove excess mucous and sacrificed. Each core was divided in two pieces; one half was fixed in liquid nitrogen and stored at −80°C for cellular diagnostic analysis, and the remaining half was fixed in either buffered, zinc-formalin (Z-Fix) for histological analysis, or in glutaraldehyde for electron microscopy analyses. One frozen sample from each tank, selected at random, was stored at the UCF and shipped to Haereticus Environmental Laboratory for analysis. The other sample was frozen at −80°C at the National Coral Research Institute (NCRI) at NSUOC. Unfortunately, the freezer lost power for several days after Hurricane Wilma so these samples are no longer useable.

Statistical analyses

We used multivariate of variance to test the general linear model

$$\{Y1_{ijkl}, Y2_{ijkl}, \ldots Yz_{ijkl}\} = \mu + \alpha_i + \beta_j + \gamma_k + I_{ij} + \ldots + \varepsilon_{ijkl}$$

where $\{Y1_{ijkl}, Y2_{ijkl}, \ldots Yz_{ijkl}\}$ was the response vector of CDS parameters from field-collected samples, μ was the grand mean of the response, α_i was the deviation due to depth, β_j was the deviation due to sewage outfall, γ_k was the deviation due to shipping inlet, I_{ij}, etc. were deviations due to interactions between the fixed effects of depth, sewage outfall, and shipping inlet, and the ε_{ijkl} were the residual errors.

We used nested analysis of covariance (ANCOVA) to examine coral responses in the laboratory experiment, using the general linear model

$$Y_{ijkl} = \mu + B_1X_1 + B_2 X_1 + \alpha_i + \beta_j + I_{ij} + D_{k(j)} + \varepsilon_{ijkl}$$

where Y_{ijkl} was the accumulation of a particular CDS parameter in lab-reared corals, μ was the grand mean of the response, B_1 and B_2 were regressors of the covariates X_1 (aisle gradient) and X_2 (door gradient), α_i was the deviation due to colony depth, β_j was the deviation due to water source, I_{ij} was the colony depth x water source interaction, $D_{k(j)}$ was the random effect of coral colony nested within depth, and the ε_{ijkl} were the residual errors. In this analysis, the main effect of colony depth was tested using a residual that contained the nested coral colony[depth] term. All other effects were tested using the residual error in the denominator. Separate nested ANCOVA's were performed for each cellular-diagnostic parameter.

We adhered to model assumptions of multivariate random, normally-distributed and independent residuals by transforming cellular-diagnostic responses as log10(x + 1). We used separate univariate tests to interpret significant MANOVA results and Tukey's Honestly Significant Difference method to separate univariate means. This procedure limited the probability of committing a Type I error even when responses were

correlated. We used hierarchical clustering to examine patterns in coral diversity, bottom cover and cellular-diagnostic responses. All statistical analyses were performed using JMP V. 4.0.4 (SAS Institute, Inc., Cary, NC, USA), with $\alpha = 0.05$ for all hypothesis tests.

Results

Percent cover of corals and associated reef organisms

Bottom substrate or *Lyngbya* was the dominant cover at all sites (Fig. 3) and these two cover types were significantly negatively correlated (Pearson's $r = -0.91$, $P < 0.002$). At each site, sponges comprised 2.4-7.1% cover, and sponge cover was positively correlated with substrate cover (Pearson's $r = 0.78$, $P < 0.03$) and negatively correlated with coverage by *Lyngbya* (Pearson's $r = -0.74$, $P < 0.04$). All sites had <4% coral cover (Fig. 4) and soft corals (gorgonians and zooanthids) varied from 1-11% cover (Appendix I). Differences in reef depth and the presence of sewage outfalls and shipping inlets did not explain significant variation in bottom composition, regardless of whether the analysis used MANOVA on original variates, on data summarized as principal components, or ANOVA on each category separately.

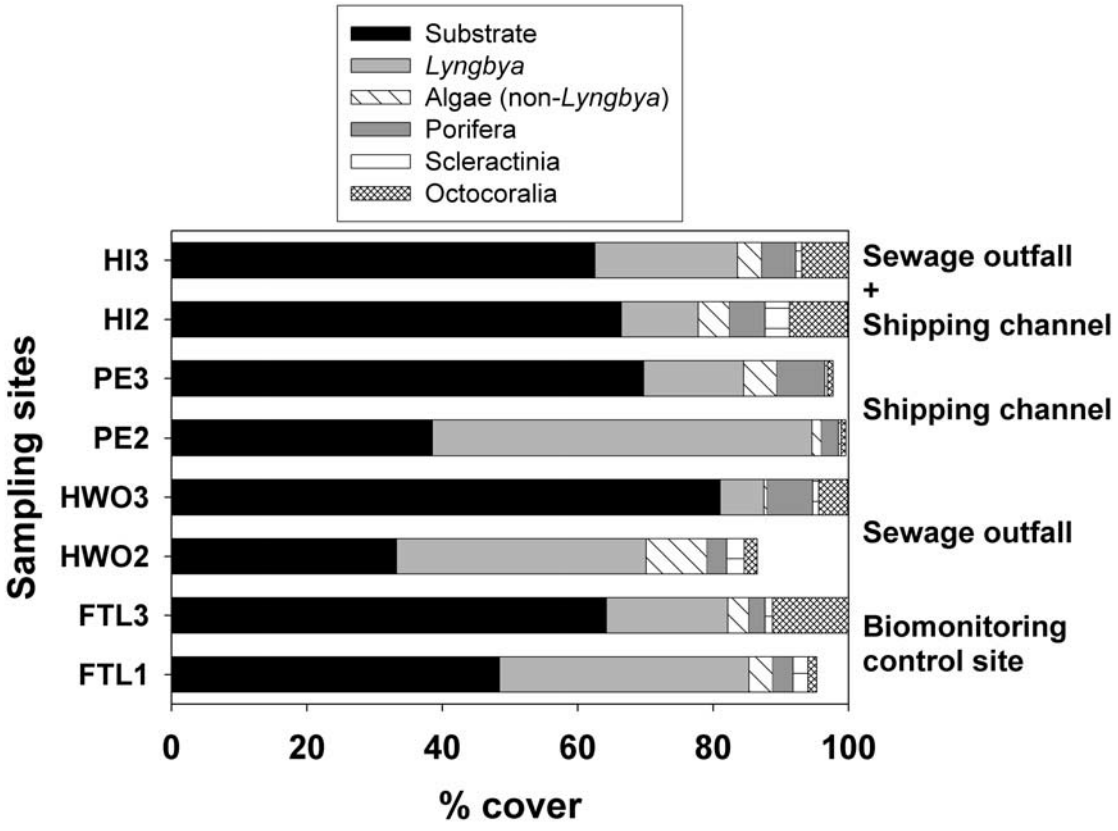

Fig. 3 Percentage bottom cover at the four paired inshore and offshore sites off Broward County, Florida.

Fig. 4. Typical bottom cover at non-biomonitoring control sites off Broward County, Florida, which were dominated by bare substrate and the invasive red alga Lyngbya. The floating white chains were used to temporarily mark locations of colonies of *Porites astreoides*. Photo by Ken Banks.

Sites clustered into two distinct groups based on bottom composition (Fig. 5). One group was comprised of three inshore sites (FTL1, PE2 and HWO2), which had the lowest percentages of bare substrate, the highest cover by *Lyngbya*, and the least cover by sponges, stony corals, and soft corals (Fig. 3). The other group comprised all four offshore sites plus the inshore HI2 site (Fig. 5). These five sites were characterized by the highest percentages of bare substrate, the lowest cover by *Lyngbya*, and the most cover by sponges, stony corals, and soft corals (Fig. 3).

Coral condition

Coral surface area varied significantly with depth (ANOVA on $\log_{10}$-transformed data: $F_{1,32} = 4.60$, $P < 0.04$) during the initial sampling period, January 26-27, 2005. On average, colonies at inshore sites had 43% less estimated surface area than colonies at offshore sites (368 ± 36.3 versus 642.3 ± 108.6 cm^2, mean ± SE). Significant differences in length (ANOVA on $\log_{10}$-transformed data: $F_{1,38} = 6.33$, $P < 0.02$) and height (ANOVA on $\log_{10}$-transformed data: $F_{1,38} = 4.47$, $P < 0.04$), but not width, contributed to this pattern.

Percentages of old and recent tissue loss varied significantly with the exact combination of sewage outfall and shipping channel at each pair of sites (significant interaction terms, Table 3) in January, 2005. Old loss of live coral tissue was significantly lower at the biomonitoring control sites than at the paired sites with just a sewage outfall (Fig. 6). The percentages of old tissue loss at the other two sites (PE and HI) were indistinguishable statistically from these two extremes. New tissue loss had a similar pattern (Fig. 6); it was significantly higher at paired sites having just a sewage

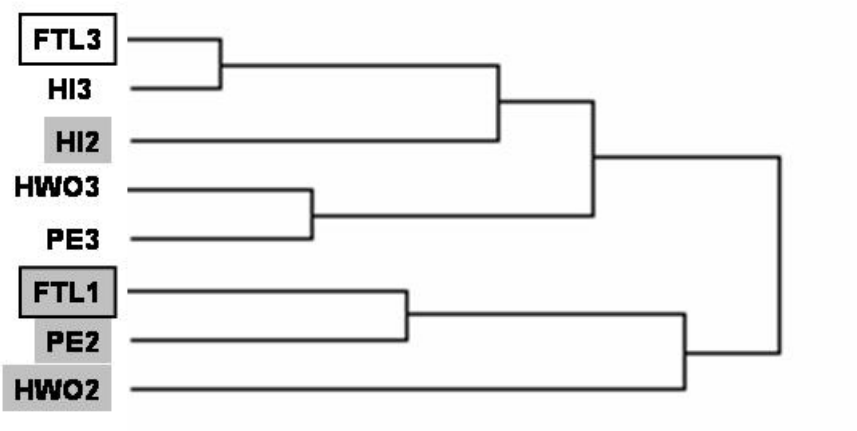

Fig. 5 Sites clustered by bottom composition, estimated as percentage cover of substrate, *Lyngbya*, other algae species beside *Lyngbya*, Porifera, Scleractinia, and Octocoralia. Shading identifies inshore sites and black boxes denote biomonitoring control sites established by the Broward County Department of Environmental Protection.

outfall (HWO) than at the other three paired sites. Most new tissue loss (Fig. 2) appeared to be caused by parrotfish or other grazers.

We observed little recent tissue loss when we resampled colonies on August 15-16, 2005. However, the percentage of total tissue loss, which includes mortality due to non-regenerated sampling lesions, varied significantly with depth, the sewage outfall x shipping channel interaction, and the three-way interaction between them (Table 4). On average, total tissue loss was low at all inshore sites and at the offshore biomonitoring control site, highest at the offshore site near a sewage outfall, and also high at the offshore sites that had shipping channels (Fig. 7). The estimate of total tissue loss at the Port Everglades offshore site (PE3) is conservative because we only re-located one colony there (Appendix II). The missing colonies must have been overturned, buried by sediment, moved off the site or destroyed. One colony at another offshore/shipping channel site (HI3) was knocked loose from the bottom and moved 30 cm from its original position near a sponge. We identified this colony from the photograph of its sampling lesion.

Lesion regeneration

Regeneration rates of biopsy lesions varied significantly with their initial area ($F_{1,34} = 7.53$, $P < 0.01$, $R^2 = 0.18$); larger lesions regenerated faster than smaller lesions (regression equation: regeneration rate [mm^2/d] = $-0.96 + 6.65 \times 10^{-3}$ x initial lesion size [mm^2]). Lesion regeneration rate also varied significantly with depth and the sewage outfall x shipping channel interaction (Table 5). Coral colonies at all inshore sites except PE had lesion regeneration rates that were significantly greater than zero (Fig. 8). In

Table 3 Results of analysis of variance on $\log_{10}$-transformed estimates of percentage old and recent tissue loss in mustard hill corals (*Porites astreoides*). Five colonies were sampled on each of four paired, inshore-offshore sites off Broward County, Florida, in January, 2005. Statistically significant terms ($\alpha = 0.05$) are in bold.

Old tissue loss

Source	df	SS	F	P
Sewage outfall (O)	1	0.3887	3.074	0.089
Shipping channel (C)	1	0.0120	0.095	0.760
D x O	1	0.0252	0.200	0.658
D x C	1	0.1473	1.165	0.289
O x C	1	0.5467	4.324	**0.046**
D x O x C	1	0.0351	0.278	0.602
Error	32	4.0462		
Total	39	5.6252		

Recent tissue loss

Source	df	SS	F	P
Depth (D)	1	0.2554	2.929	0.097
Sewage outfall (O)	1	0.7080	8.121	**0.008**
Shipping channel (C)	1	0.8048	9.232	**0.005**
D x O	1	0.0832	0.955	0.336
D x C	1	0.2859	3.279	0.080
O x C	1	1.4372	16.487	**0.0003**
D x O x C	1	0.0047	0.054	0.817
Error	32	2.7895		
Total	39	6.3687		

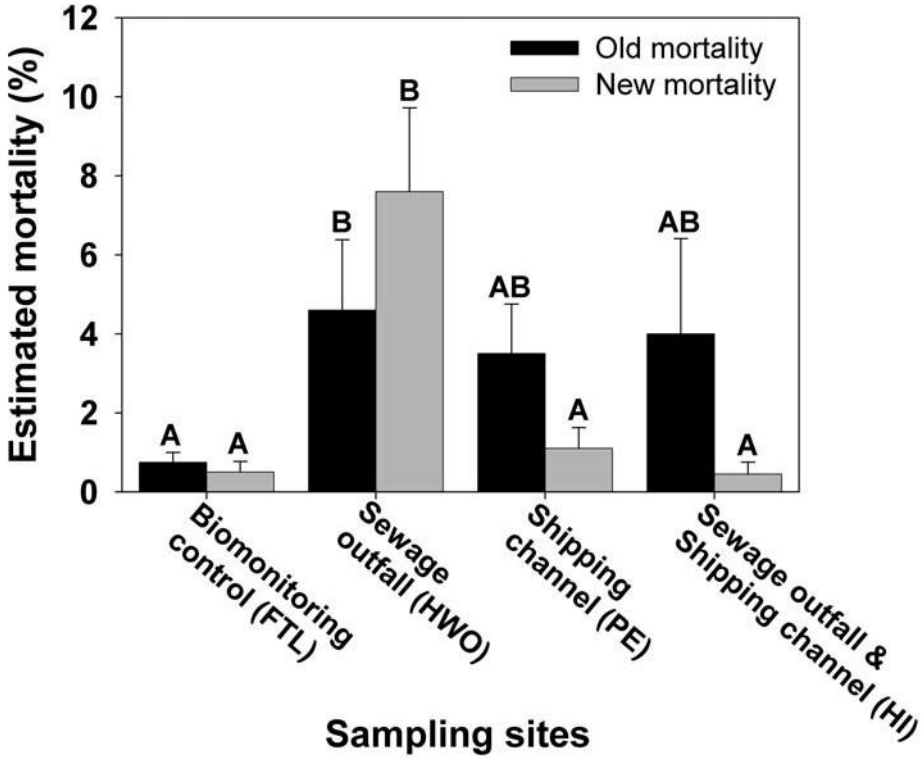

Fig. 6 Estimated percentages of old and recent tissue loss on mustard hill corals (*Porites astreoides*) at four locations off Broward County, Florida. Data are untransformed means ± 1 SE; N = 10 colonies at each location, sampled January 26-27, 2005. Levels of old or recent tissue loss that are not connected by the same letter differed significantly in ANOVA on $\log_{10}(x+1)$-transformed variates.

Table 4 Results of analysis of variance on $\log_{10}$-transformed estimates of percentage total tissue loss on mustard hill corals (*Porites astreoides*). Up to five colonies were re-sampled on each of four paired, inshore-offshore sites off Broward County, Florida, in August, 2005. Statistically significant terms ($\alpha = 0.05$) are in bold. Model $R^2 = 0.59$.

Source	df	SS	F	**P**
Depth (D)	1	0.3554	20.485	**0.0001**
Sewage outfall (O)	1	0.0001	0.003	0.954
Shipping channel (C)	1	0.0263	1.520	0.228
D x O	1	0.0166	0.960	0.336
D x C	1	0.0039	0.225	0.639
O x C	1	0.1802	10.386	**0.003**
D x O x C	1	0.1313	7.565	**0.010**
Error	28	0.4858		
Total	35	1.1958		

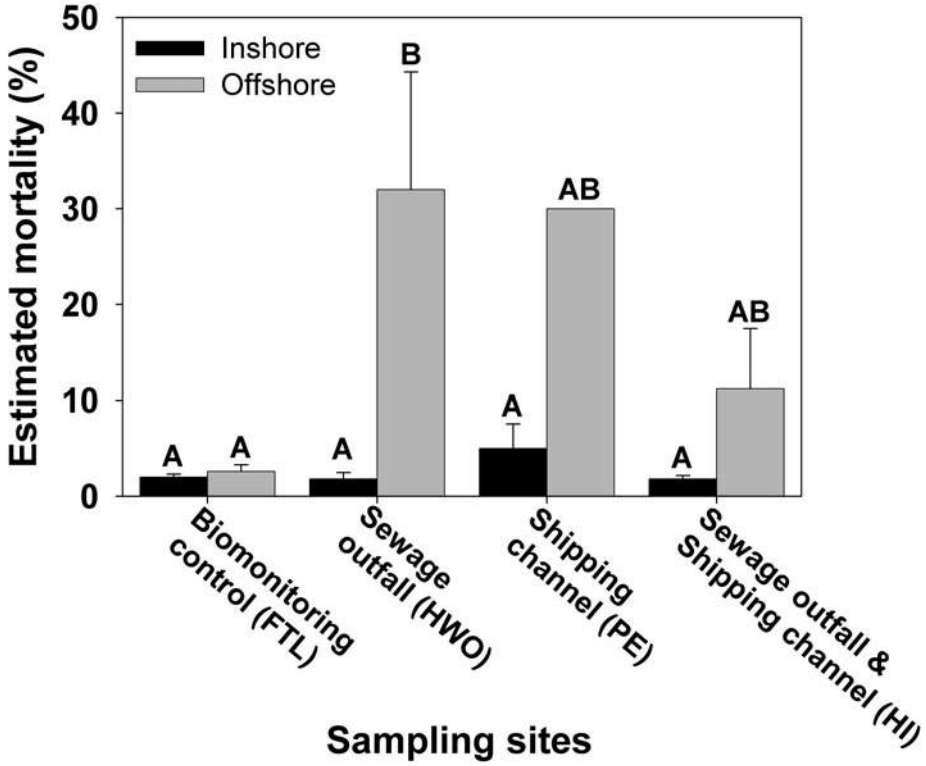

Fig. 7 Estimated percentages of total tissue loss on mustard hill corals (*Porites astreoides*) at four paired sites off Broward County, Florida. Data are untransformed means ± 1 SE; N = 1-5 colonies at each location, sampled August 14-15, 2005. Levels that are not connected by the same letter differed significantly in ANOVA on square-root transformed variates.

Table 5 Results of analysis of covariance on regeneration rates of mustard hill corals (Porites astreoides). Up to five colonies were re-sampled at each of four paired, inshore- offshore sites off Broward County, Florida, in August, 2005. Statistically significant terms (α = 0.05) are in bold. Model R2 = 0.60.

Source	df	SS	F	P
Initial lesion size	1	3.4202	11.40	**0.002**
Depth (D)	1	4.4854	14.95	**0.001**
Sewage outfall (O)	1	0.0041	0.014	0.908
Shipping channel (C)	1	0.2244	0.813	0.375
D x O	1	0.4213	1.404	0.246
D x C	1	0.0021	0.007	0.934
O x C	1	1.3334	4.444	**0.044**
D x O x C	1	0.6497	2.165	0.153
Error	27	8.1031		
Total	35	20.0190		

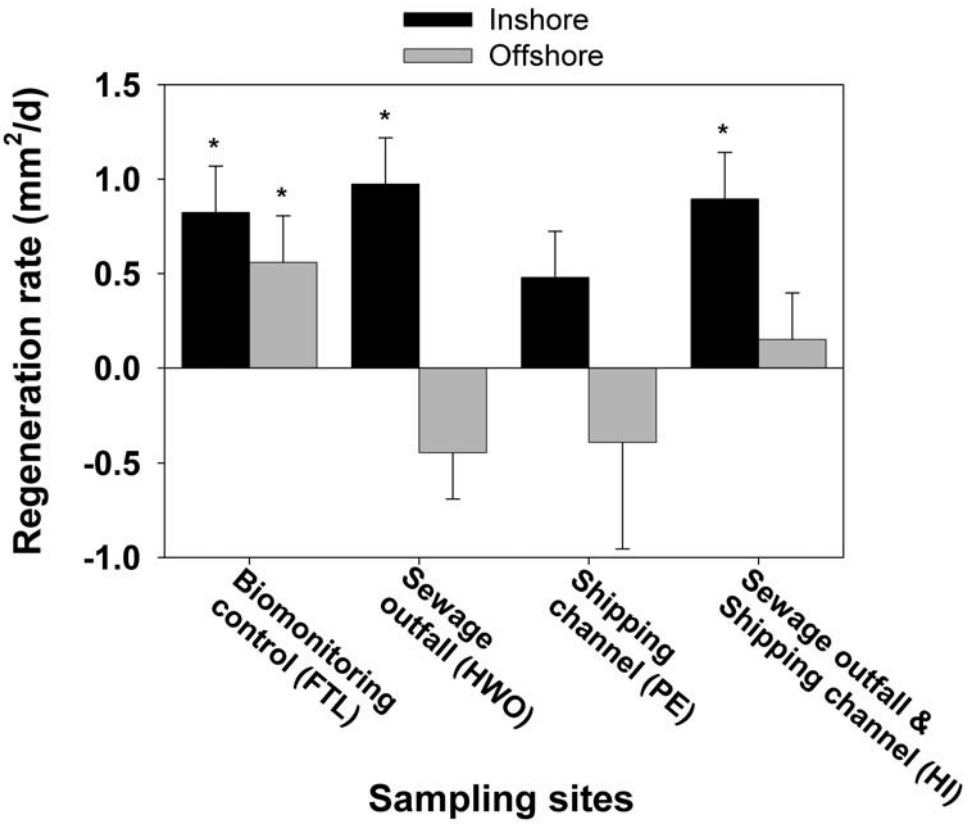

Fig. 8 Regeneration rates (mm^2/d) of mustard hill corals (*Porites astreoides*) at four paired sites off Broward County, Florida. Data are least squares means (± 1 SE) from analysis of covariance, with initial lesion size as the covariate. Asterisks signify regeneration rates that differed significantly from zero. N = 1-5 colonies at each location.

contrast, among the offshore sites only colonies at the biomonitoring control site were significantly greater than zero (Fig 9) . Regeneration rates at the three other offshore sites were indistinguishable statistically from zero; mean regeneration rates at the offshore sewage outfall (HWO3) and shipping channel (PE3) were negative (Fig. 8).

Cellular-diagnostic responses of biopsied corals

Multivariate and univariate analyses

The main effects of depth (MANOVA: $F_{10,20} = 5.48$, $P < 0.001$) and sewage outfall (MANOVA: $F_{10,20} = 4.38$, $P < 0.003$), and the depth x shipping channel (MANOVA: $F_{10,20} = 2.47$, $P < 0.04$) and sewage outfall x shipping channel (MANOVA: $F_{10,20} = 3.35$, $P < 0.02$) interactions all explained significant variation in the vector of ten cellular-diagnostic responses. Eight parameters contributed most to the significant multivariate response: Hsp 60, Grp 75, ubiquitin, CYP 2, CYP 6, FC, MDR and GST. Accumulations of sHsp and Cu/Zn SOD did not vary significantly with depth or proximity to potential land-based sources of pollution (Appendix III).

Heat shock protein 60 and ubiquitin comprised assays of protein metabolic condition. Averaged across all sites, offshore corals had significantly more Hsp 60 than did inshore corals (ANOVA $F_{1,29} = 9.00$, $P < 0.006$) and colonies from HI had less Hsp 60 than expected for a site near both a sewage outfall and shipping channel (significant interaction term: ANOVA $F_{1,29} = 10.57$, $P < 0.003$; Fig. 10A). Ubiquitin levels varied significantly with the depth x shipping channel interaction (ANOVA $F_{1,29} = 4.51$, $P < 0.043$). Corals at inshore sites without a nearby shipping channel had twice as much

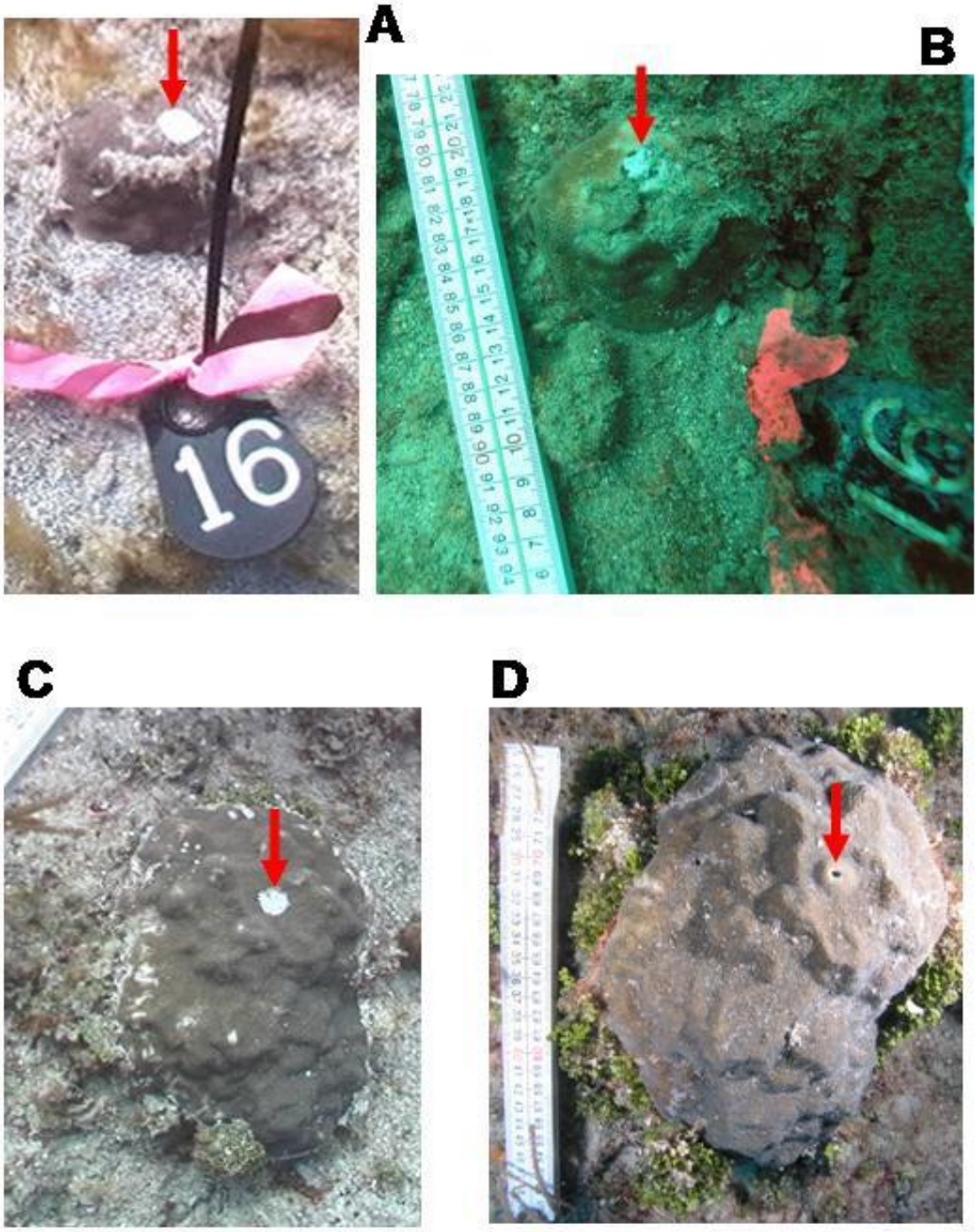

Fig. 9. Sampling lesion on colony 16 from PE2: A) initial, and B) expanding, thereby increasing the total area of tissue loss. Sampling lesion of colony 33 from FTL3: A) initial, B) almost totally regenerated.

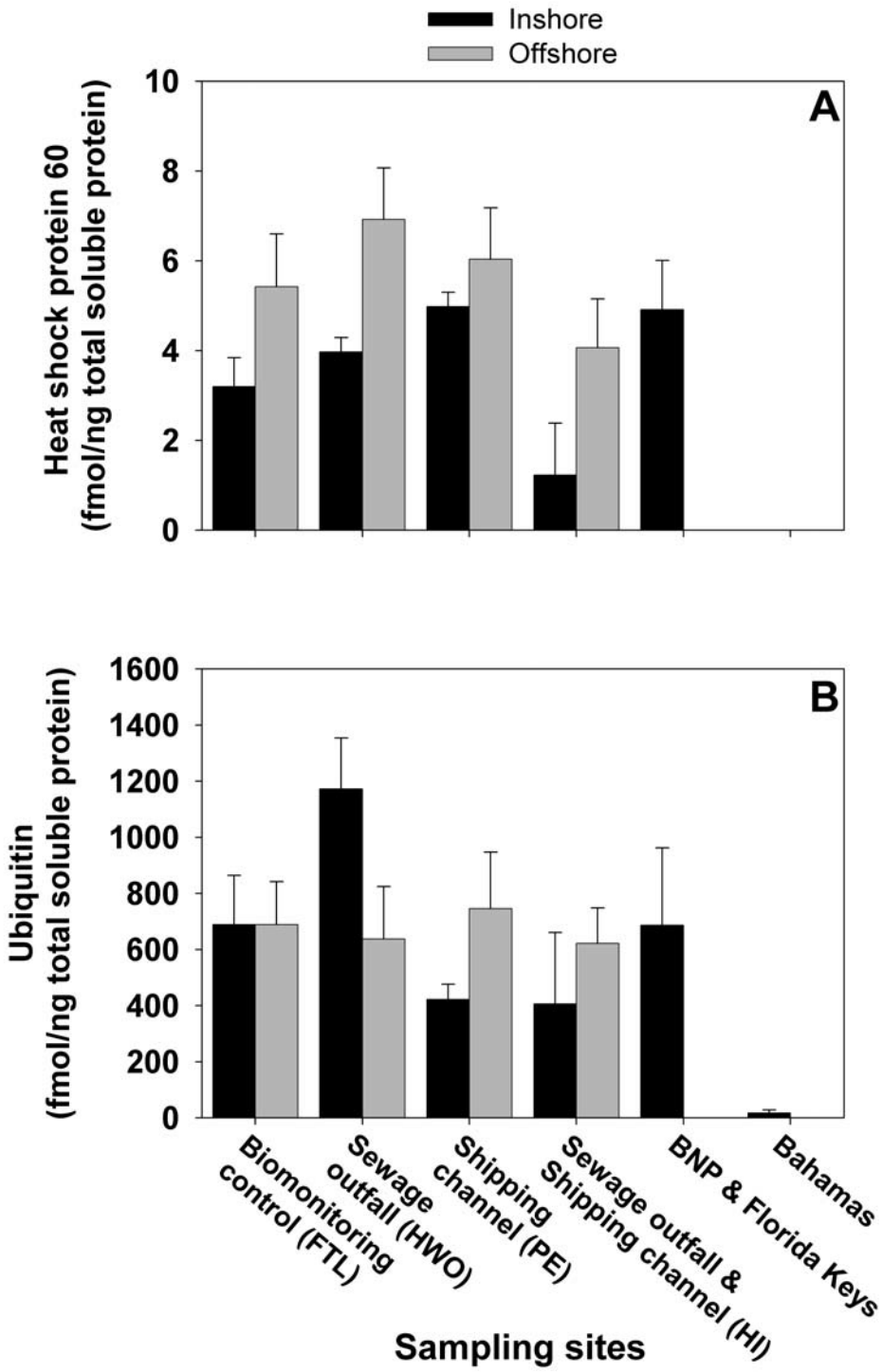

Figure 10 Mean (± 1 SE) accumulations of parameters indicative of protein metabolic condition in *Porites astreoides* sampled off Broward County, Florida, and two other regions. A) Heat shock protein 60, B) ubiquitin. Abbreviations are as in the text.

ubiquitin as colonies at inshore sites with a shipping channel nearby (Fig. 10B). In contrast, corals at offshore sites had intermediate ubiquitin levels.

Total sHsp's and Grp 75 assayed of overall metabolic condition. Total sHsp levels did not vary among the Broward County sites (Fig. 11A). Levels of Grp 75 varied significantly with the sewage outfall x shipping channel interaction (ANOVA $F_{1,29}$ = 15.79, P = 0.0004). Averaged across both depths, corals at HWO had more than three times as much Grp 75 as colonies at the biomonitoring and HI sites (difference significant: Tukey's HSD test; Fig. 11B).

Copper/zinc superoxide dismutase and FC comprised assays of oxidative stress and response. Levels of Cu/Zn SOD did not vary significantly among the Broward County sites (Fig. 12A). In contrast, FC levels varied significantly with the depth x sewage outfall interaction (ANOVA $F_{1,29}$ = 5.86, P < 0.03). Corals at inshore sites with a sewage outfall nearby had significantly less FC than colonies at inshore sites without a sewage outfall and offshore sites with a sewage outfall nearby. Offshore colonies at sites without a sewage outfall were indistinguishable statistically from these two groups (Fig. 12B).

Cytochrome P450-2 class, CYP 6, GST and MDR assayed coral responses to xenobiotics. Responses of CYP 2 and GST were very similar (Fig. 13A,B); both parameters were significantly higher at offshore than at inshore sites (ANOVA's: $F_{1,29}$ > 6.17, P < 0.02). Mean CYP 6 levels were significantly higher at sites without shipping channels compared to sites near them (ANOVA: $F_{1,29}$ = 4.39, P = 0.045). On average, sites without shipping channels had CYP 6 levels 26% higher than sites with shipping channels (Fig. 13C). Mean levels of MDR varied significantly with depth, sewage

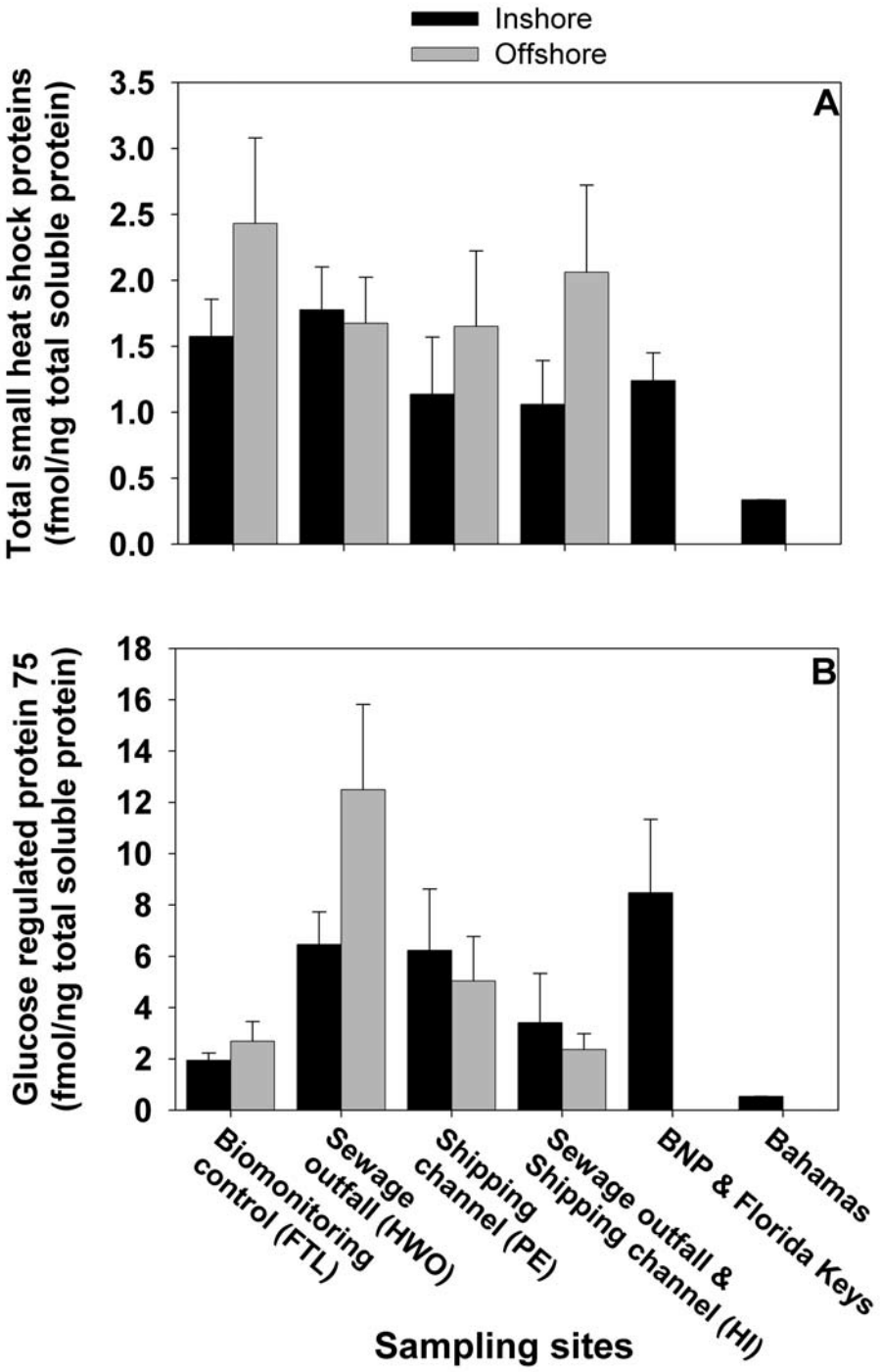

Figure 11 Mean (± 1 SE) accumulations of parameters indicative of cellular metabolic condition in *Porites astreoides* sampled off Broward County, Florida, and two other regions. A) Total small heat shock proteins, B) glucose-regulated protein. Abbreviations are as in the text.

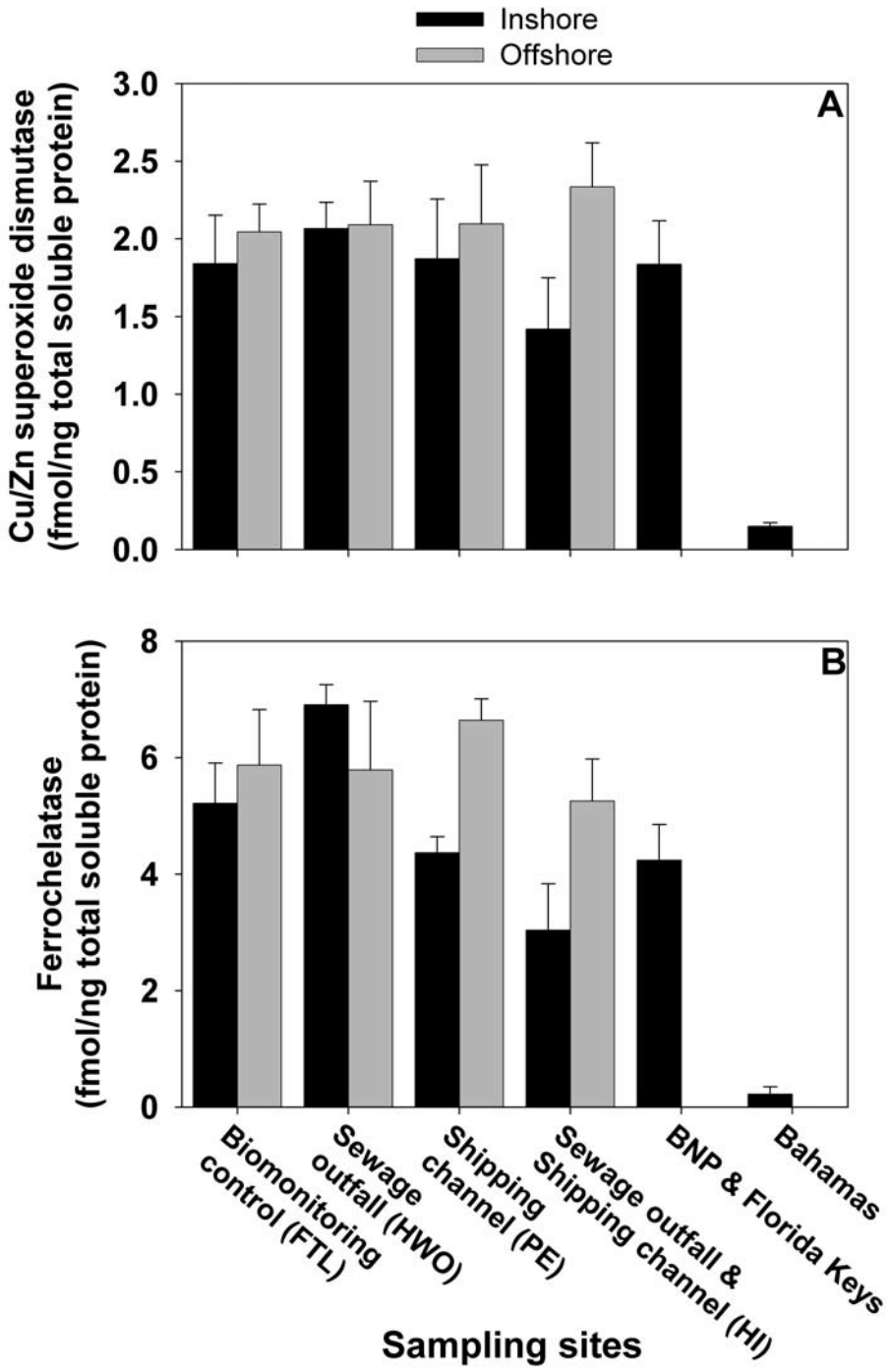

Figure 12 Mean (± 1 SE) accumulations of parameters indicative of oxidative stress response in *Porites astreoides* sampled off Broward County, Florida, and two other regions. A) Cu/Zn superoxide dismutase, B) ferrochelatase. Abbreviations are as in the text.

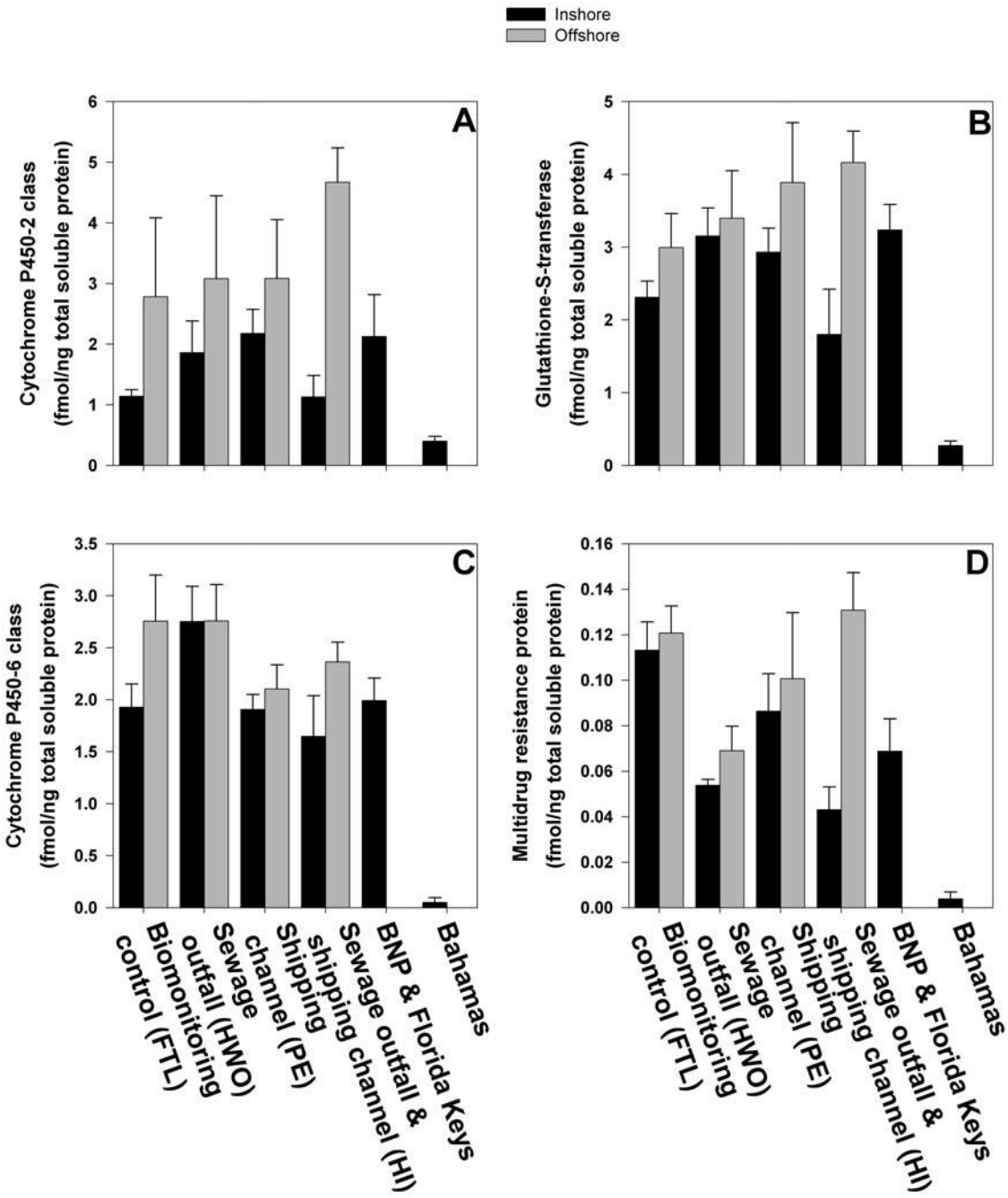

Figure 13 Mean (± 1 SE) accumulations of parameters indicative of xenobiotic response in *Porites astreoides* sampled off Broward County, Florida, and two other regions. A) Cytochrome P450-2 class, B) glutathione-S-transferase, C) Cytochrome P450-6 class, and D) multidrug resistance protein. Abbreviations are as in the text.

outfall, and the sewage outfall x shipping channel interaction (ANOVA: all $F_{1,29} > 4.97$, $P < 0.034$). On average, mean MDR levels were 26% lower on inshore sites compared to offshore sites, and 30% lower at sites near sewage outfalls compared to those without them (Fig. 13D). In addition, mean MDR levels were significantly higher at the FTL biomonitoring sites than at the two HWO sites (0.117 ± 0.00820 versus 0.061 ± 0.00531 fmol/pg TSP, respectively). Mean MDR levels at the paired PE and HI sites were statistically indistinguishable from both of these groups (Tukey's HSD test).

Comparisons with other sites

Mean accumulations of every cellular-diagnostic parameter at the eight Broward County sites were indistinguishable statistically from those of *P. astreoides* sampled in Biscayne National Park and the Florida Keys (one-way ANOVA followed by Tukey's HSD test). However, mean accumulations of all biomarkers at every U.S. site were significantly higher than those of *P. astreoides* from the Bahamas (Figs. 10-13). The only exception was Hsp 60, which was undetectable in Bahamian corals and did not differ significantly from zero in inshore HI colonies.

Cluster analysis confirmed that corals from the Bahamas had very different cellular-diagnostic responses than corals sampled in BNP, FKNMS, and off Broward County (Fig. 14). The two Hollywood sewage outfall sites clustered with FKNMS corals; the remaining inshore and offshore sites formed natural groups (Fig. 14).

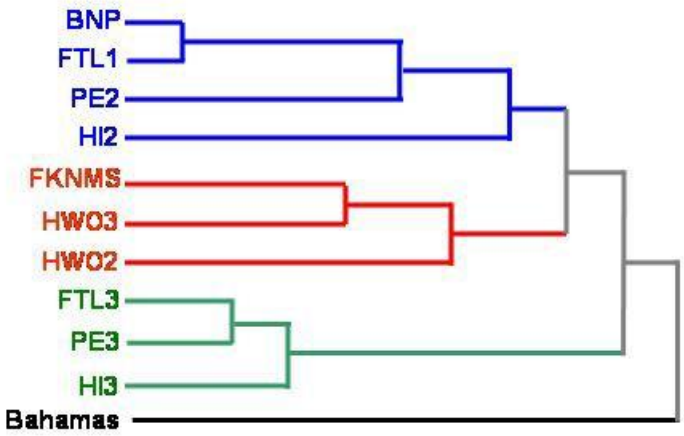

Fig. 14 Sites clustered by mean cellular-diagnostic responses into four groups. Corals from the Bahamas had low levels of all parameters. The two stations near the City of Hollywood's sewage outfall (HWO2 and HWO3) group with colonies sampled in Florida Keys National Marine Sanctuary (Molasses Reef, Looe Key, and Marquesas Atoll). The remaining three inshore (blue) and offshore sites (green) cluster together. The former group also includes corals sampled at Biscayne National Park (BNP).

Cellular-diagnostic responses of corals in the laboratory experiment

After adjusting for the two-dimensional gradient in the laboratory, mean MDR accumulations were significantly higher in coral explants exposed to artificial sea water compared to PE water (least-squares means and SE's of $\log_{10}(MDR + 1)$: for artificial sea water, 0.034 ± 0.00226; for PE water, 0.026 ± 0.00225; $F_{1,16} = 5.55$, $P < 0.032$). Mean accumulations of every other CDS parameter did not differ significantly between treatments or depths (Appendix IV). Instead, mean accumulations of all CDS parameters except sHsp differed significantly between coral colonies nested within depth (Table 6). There was substantial genetic variation (up to 90% of total phenotypic variation) between coral colonies collected within inshore and offshore sites, particularly in mean accumulations of GST, Cu/Zn SOD, ubiquitin, Hsp 60, MDR and CYP 6. Genetic variation between colonies nested within depths was moderate for GRP 75 and FC (56 and 49%, respectively), and extremely low (1%) for sHsp. When the colony nested within depth term was removed from statistical models, all except sHsp showed significant differences in mean CDS levels between depths, which was consistent with the pattern observed in field samples.

Correlated responses

Developing prognostic indicators of coral condition requires linking parameters of molecular and cellular function with the fitness of individuals and ecosystem structure and function (Depledge et al. 1993, Moore 2001, Fauth et al. 2003). In *Porites astreoides* sampled off Broward County, percent tissue loss regressed positively on GRP 75 (mortalin) accumulation (Fig. 15), which is essential for cell proliferation. Backward

Table 6 Results of analysis of covariance on cellular-diagnostic parameters of mustard hill corals (*Porites astreoides*) from inshore and offshore biomonitoring control sites exposed to artificial sea water or water from Port Everglades, Florida.

Source of variation	GST	sHsp	Cu/ZN SOD	Ubiquitin	GRP 75
Door gradient	3.76	0.08	5.43*	2.41	10.00
Aisle gradient	0.48	2.00	1.45	3.12	2.52
Water	0.02	0.89	0.63	0.86	0.02
Colony depth	2.55	2.89	2.61	0.33	4.32
Colony[Colony depth]	13.4****	0.86	18.1****	28.8****	4.44*
Water*Colony depth	1.39	1.98	0.100	0.17	0.23
Model R^2	0.903	0.380	0.902	0.878	0.817
% variation genetic	87.4	1.04	87.2	87.1	56.2

*P < 0.05; ****P < 0.0001

Table 6 continued.

Source of variation	Hsp 60	MDR	FC	CYP 6
Door gradient	4.33	10.6**	5.99*	0.9
Aisle gradient	2.12	5.01*	2.79	1.27
Water	1.06	5.63*	1.08	0.47
Colony depth	4.03	2.01	1.58	0
Colony[Colony depth]	18.6****	34.4****	5.81*	8.84**
Water*Colony depth	0.02	0.29	0.45	1.66
Model R^2	0.935	0.896	0.658	0.640
% variation genetic	90.6	87.0	49.1	61.1

*P < 0.05; **P < 0.01; ****P < 0.0001

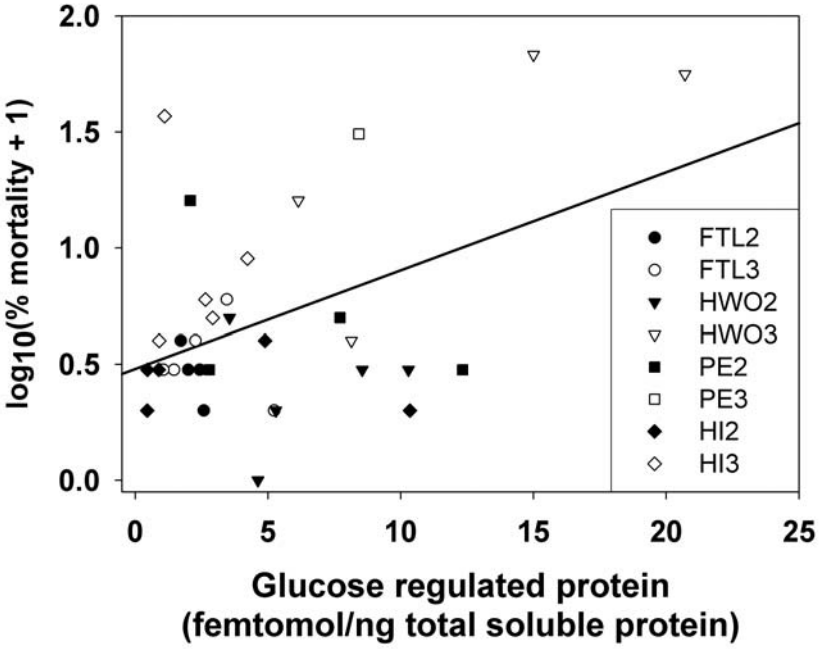

Fig. 15 Log-linear regression of coral % mortality as a function of the concentration of glucose regulated protein 75 (Grp 75), which also is known as mortalin. Regression equation: Log10(% mortality + 1) = 0.48 + 0.042 Grp 75 (femtomoles/ng total soluble protein). $F_{1,32} = 8.10$, $P < 0.008$, $R^2 = 0.20$.

Table 7 Regression model relating $\log_{10}$(percentage mortality + 1) of *Porites astreoides* as a function of cellular-diagnostic parameters. Statistically significant regressors are in bold. Whole model $F_{4,29} = 4.78$, $P < 0.005$, $R^2 = 0.40$.

Source	Estimate	Std. error	t-ratio	P
Intercept	1.32	0.5521	2.39	**0.0237**
Log_{10}(Grp + 1)	0.419	0.2108	1.99	0.0565
Log_{10}(ubiquitin + 1)	-0.845	0.2564	-3.30	**0.0026**
Log_{10}(CYP 2 + 1)	0.636	0.3241	1.96	0.0593
Log_{10}(CYP 6 + 1)	2.150	0.7130	3.01	**0.0053**

Table 8 Log-linear regression model of the regeneration rate (mm/d) of *Porites astreoides* as a function of cellular-diagnostic parameters. Statistically significant regressors are in bold. Whole model $F_{3,33} = 3.00$, $P < 0.046$, $R^2 = 0.24$.

Source	Estimate	Std. error	t-ratio	P
Intercept	-0.0907	1.015	-0.09	0.9294
Log_{10}(ubiquitin + 1)	1.03	0.471	2.20	**0.0359**
Log_{10}(CYP 2 + 1)	-1.32	0.596	-2.22	**0.0342**
Log_{10}(CYP 6 + 1)	-3.14	1.284	-2.44	**0.0207**

stepwise selection identified a log-log model with Grp 75, ubiquitin, Cyp 2 and CYP 6 as predictors of percent tissue loss (Table 7). Coral colonies with low ubiquitin levels and high levels of Grp 75, CYP 2, and CYP 6 lost the most tissue. Similarly, levels of ubiquitin, Cyp 2 and CYP 6 were significant predictors of lesion regeneration rate (Table 8). Coral colonies with high ubiquitin levels and low levels of CYP 2 and CYP 6 had the highest regeneration rates.

Discussion

The objective of this pilot project was to test the feasibility of using cellular diagnostics to link land-based sources of pollution with coral reef degradation. Our research detected elevated biomarker levels in tissue samples of mustard hill coral (*Porites astreiodes*) collected at four paired inshore and offshore stations off Broward County, FL, which included biomonitoring control sites and sites near a sewage outfall, an inlet mouth, and a sewage outfall adjacent to an inlet mouth. We used standard ecological methods to assess coral health, including monitoring regeneration of lesions that we created while sampling colonies. The results show that coral colonies at different sites varied in tissue loss and regeneration rate, which in turn were associated with specific changes in cellular-diagnostic parameters.

Typical coral monitoring programs use temporal changes in the percentage of substrate occupied by live coral tissue as an indicator of reef health (Gardner et al. 2003). This method requires increasingly greater sampling effort to detect significant changes as coral cover approaches zero. At our four paired sites off Broward County, mean coral

cover was <4%, making this method imprecise and impractical. Instead we used cluster analysis to determine that three inshore stations (FTL 1, PE2 and HWO2) had the lowest percentages of bare substrate and cover by corals and sponges. In contrast, the four offshore stations and the inshore Hillsborough Inlet station (HI2) grouped together and had the highest percentages of bare substrate, corals and sponges, and the least area with the cyanobateria *Lyngbya*. The area near the mouth of Hillsborough Inlet may present a challenging environment for *P. astreoides*; the species was absent from the first area that we dove and we had to explore further south. The dichotomy between inshore and offshore stations is a repeated theme in the data, and illustrates the insight provided by multivariate analyses of coral reef community structure.

Colonies at offshore stations were significantly longer and higher than those at inshore stations and also had higher percentages of total tissue loss and lower regeneration rates. The exception was the offshore FTL3 biomonitoring control site, where coral colonies had little tissue loss and high regeneration rates. Total tissue loss was greatest near the City of Hollywood's sewage outfall and moderately high at the offshore Port Everglades and Hillsborough Inlet stations. These three stations also had lesion regeneration rates that were indistinguishable from zero, and in two cases (HWO3 and PE3) tended to be negative; lesions grew larger instead of healing. The inability to regenerate small lesions indicates that conditions were poor for coral growth and reproduction (e.g., Lester and Bak 1985; Meesters et al. 1997; Mascarelli and Bunkley-Williams 1999; Fine et al. 2002) when we sampled colonies in January, 2005.

Cellular-diagnostic parameters were elevated at all four paired stations and at several other sites that we sampled the following week in Biscayne National Park and

Florida Keys National Marine Sanctuary, compared to two coral colonies sampled in the remote Exhumas, Bahamas. This pattern was clear and consistent and illustrates how much easier it is to identify patterns when at least one site is distant from major sources of anthropogenic stress. Cluster analysis organized sites into four main groups: Bahamas, Hollywood outfall sites + FKNMS, the remaining offshore sites, and the three remaining inshore sites + Biscayne National Park. *Porites astreoides* from the Bahamas had very low accumulations of all cellular-diagnostic parameters, and total sHsp was below detection limits there. This enzyme localizes to the mitochondria in cnidarians and other invertebrates, where it protect aspects of oxidative phosphorylation (Morrow et al., 2000). High levels of cnidarian sHsp at all eight Broward County stations indicate they were responding to an oxidative stress (Downs et al. 2006).

The two stations near the City of Hollywood's sewage outfall and sites in Florida Keys National Marine Sanctuary were characterized by high levels of GRP 75, also known as mortalin. This enzyme is induced by glucose deprivation and is involved in pathways to cellular senescence and transformation. In our study, elevated GRP 75 levels were associated with increased amounts of coral tissue loss. Colonies at these sites also had high levels of ubiquitin, which tags damaged proteins for degradation. Combined, these results suggest that coral nutrition was altered by sewage discharge and resulted in higher than normal rates of protein turnover. Nutrient enrichment and sediment loads from sewage discharge are known to directly and indirectly inhibit growth and recruitment of corals and decrease coral cover, including in the Florida Keys (e.g., Pastorak and Bilyard 1985, LaPointe et al. 2004).

Three offshore sites (FTL3, PE3 and HI3) were characterized by high levels of Hsp 60, CYP 2 and MDR. Elevated levels of these three parameters are consistent with oxidative damage caused by exposure to xenobiotics. Cytochrome P450-2 class is induced by electrophilic carcinogens, drugs, and other environmental pollutants, which it oxidizes in a cellular suicide reaction. Glutathione-S-transferase, which also was elevated at these sites, conjugates the oxidized xenobiotic to glutathione, which is pumped from the cell by MDR. Multidrug resistance protein is an ABC cassette protein that lowers the intracellular concentration of toxic compounds below their level of toxicity (Bard 2000). Increases in MDR usually occur only in response to an organic xenobiotic (Bard, 2000; Sauna et al. 2001). The antibody used in this study binds both the cnidarian and dinoflagellate isoforms, hence results are a composite of MDR expression in both. Together, these results are consistent with offshore corals at the FTL3 biomonitoring site and off Port Everglades and Hillsborough Inlet reacting to exposure to anthropogenic contaminants. Regeneration of sampling lesions was negatively correlated with elevated levels of CYP 2, which suggests that mounting xenobiotic defenses had a metabolic cost: impaired ability to repair tissue damage. We plan to analyze frozen sediment and water samples to identify likely stressors at these sites.

Coral colonies at three inshore stations (FTL1, PE2 and HI2) and Biscayne National Park were characterized by cellular-diagnostic responses that tended to be lower than at the other Broward County sites. Corals at these three inshore stations also had moderate to high regeneration rates and little tissue loss, which is consistent with the defense trade-off hypothesis. While seemingly contrary to conventional wisdom, nearshore patch reefs in the Florida Keys have lost less live coral cover than more

offshore reef communities (Porter et al. 2001). On such inshore reefs, coral colonies appear to be in better condition than conspecifics on offshore reefs, in part because inshore colonies accumulate defensive compounds more rapidly and return to homeostasis quickly once stressors recede (e.g., Downs et al. 1999; Fauth 2004; Downs et al. in press). Much of the difference between inshore and offshore corals may have an ecotypic basis, as differences between coral clones were strongly expressed in the laboratory experiment even after two months of acclimation.

The laboratory experiment tested whether Port Everglades water induced cellular-diagnostic responses similar to those of corals samples offshore. We found no evidence that water collected at high tide from Port Everglades induced coral stress responses compared to the artificial seawater control. This suggests that contaminants (if present) must be bioaccumulated, combined with other stressors, or act indirectly (e.g., by increasing the competitive effects of algae and invertebrates) to impair corals. Alternatively, contaminant release may be episodic, tidal, or seasonal; the single sampling event that we conducted cannot detect temporal variation in coral responses. Reciprocal transplants or common garden experiments using coral colonies from the more "pristine" and impacted sites would be useful for evaluating longer-term coral responses to environmental stress. Combined with water quality and contaminant analyses conducted at the same time and place, these experiments can strengthen the chain of causality between land-based sources of pollution and coral reef degradation.

Porites astreoides is considered a hardy, r-selected or weedy coral species that is capable of persisting under conditions that kill less-tolerant species. Consequently, cellular-diagnostic parameters are generally induced less under environmental stress in *P*.

astreoides compared to members of the *Montastraea annularis* species complex (Fauth and Downs, unpublished data from Flower Garden Banks National Marine Sanctuary and the Florida reef tract). Greatly elevated levels of cellular-diagnostic parameters and low coral cover at all sites we sampled off Broward County are cause for concern, especially when regeneration rates were indistinguishable from zero at 4 of 8 stations. Observations made during sampling suggest that these reef habitats were more luxuriant in the past twenty years than at present. Our preliminary study begins establishing a line of evidence suggesting that land-based sources of pollution are affecting the status and trends of these communities. While this line of inquiry is in its infancy, it suggests that the reefs supporting vital elements of Southeast Florida's tourist and recreation economies (Leeworthy and Bowker 1997) and should receive greater protection from potential damage due to land-based sources of pollution.

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Appendix I

Estimated percent cover of substrate, corals and other benthic taxa at four paired stations off Broward County, Florida.

Species / group	HWO2A		HWO3		PE2		PE3	
	mean	se	mean	se	mean	se	mean	se
Substrate	33.34	3.27	81.11	1.14	38.65	1.31	69.82	1.48
Porites asteroides	0.16	0.10	0.06	0.06	0.06	0.06	0.00	0.00
Favia fragum	0.00	0.00	0.00	0.00	0.06	0.06	0.00	0.00
Porites porites	0.00	0.00	0.06	0.06	0.00	0.00	0.00	0.00
Siderastrea siderea	0.06	0.06	0.00	0.00	0.12	0.07	0.11	0.06
Montastraea cavernosa	0.62	0.62	0.15	0.09	0.00	0.00	0.29	0.07
Meandrina meandrites	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acropora cervicornis	0.36	0.29	0.00	0.00	0.00	0.00	0.00	0.00
Dichocoenia stokesii	0.22	0.22	0.00	0.00	0.00	0.00	0.00	0.00
Stephanocoenia michelinii	0.00	0.00	0.06	0.06	0.00	0.00	0.00	0.00
Diploria labyrinthiformis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Diploria strigosa	0.68	0.55	0.45	0.27	0.00	0.00	0.00	0.00
Siderastrea radians	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Scleractinia	0.00	0.00	0.06	0.06	0.00	0.00	0.04	0.04
Colpophyllia natans	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Solenastrea bournoni	0.00	0.00	0.00	0.00	0.12	0.12	0.00	0.00
Porifera	2.93	0.87	6.73	1.59	2.54	0.44	7.13	0.84
zoanthidae (*Palythoa*)	13.42	2.76	0.00	0.00	0.36	0.29	2.26	1.76
Octocoral	1.87	0.63	4.27	0.91	0.72	0.36	0.83	0.38
Dictyota	0.12	0.07	0.00	0.00	0.00	0.00	0.00	0.00
Halimeda	0.00	0.00	0.06	0.06	0.00	0.00	2.30	1.35
Lobophora	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.04
Lyngbya	36.84	5.80	6.40	0.96	56.00	2.38	14.67	0.55
Padina	0.11	0.11	0.00	0.00	0.00	0.00	0.00	0.00
Macroalgae	0.67	0.32	0.13	0.07	1.30	0.65	0.36	0.25
Algal mat community	7.97	3.79	0.30	0.11	0.06	0.06	2.16	0.55
Crustose coralline algae	0.17	0.10	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL	100.00	0.00	99.88	0.00	100.00	0.00	100.00	0.00

Species / group	HI2		HI3		FTL1		FTL3	
	mean	se	mean	se	mean	se	mean	se
Substrate	66.53	1.75	62.64	5.88	48.49	3.14	64.27	2.95
Porites asteroides	0.37	0.22	0.31	0.19	0.24	0.16	0.13	0.08
Favia fragum	0.06	0.06	0.07	0.07	0.08	0.08	0.00	0.00
Porites porites	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.07
Siderastrea siderea	0.27	0.01	0.00	0.00	0.00	0.00	0.27	0.19
Montastraea cavernosa	1.13	0.38	0.31	0.19	0.86	0.57	0.26	0.26
Meandrina meandrites	0.37	0.37	0.05	0.05	0.07	0.07	0.00	0.00
Acropora cervicornis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dichocoenia stokesii	0.06	0.06	0.00	0.00	0.14	0.14	0.00	0.00
Stephanocoenia michelinii	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Diploria labyrinthiformis	0.28	0.28	0.00	0.00	0.00	0.00	0.00	0.00
Diploria strigosa	0.14	0.14	0.00	0.00	0.00	0.00	0.00	0.00
Siderastrea radians	0.00	0.00	0.05	0.05	0.00	0.00	0.35	0.35
Scleractinia	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.07
Colpophyllia natans	0.90	0.45	0.11	0.11	0.00	0.00	0.00	0.00
Solenastrea bournoni	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Porifera	5.34	0.79	4.97	1.35	3.01	0.85	2.38	0.64
zoanthidae (*Palythoa*)	0.14	0.08	0.00	0.00	4.75	0.83	0.00	0.00
Octocoral	8.58	0.78	6.88	2.03	1.34	0.56	11.25	2.41
Dictyota	0.00	0.00	0.00	0.00	0.77	0.33	0.00	0.00
Halimeda	0.27	0.27	0.00	0.00	0.15	0.09	0.00	0.00
Lobophora	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lyngbya	11.25	1.35	21.04	2.77	36.75	2.17	17.88	1.24
Padina	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Macroalgae	2.42	0.25	1.46	0.45	1.20	0.38	1.31	0.91
Algal mat community	1.56	0.25	2.11	0.32	1.35	1.35	1.77	0.88
Crustose coralline algae	0.33	0.15	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00

Appendix II

Ecological aspects of *Porites* colonies.

Site	Coral #	depth (m)	initial lesion size mm^2	regen rate (mm^2/d)	total tissue loss (%)
HWO3	1	14.5	176.8	-0.6059701	67
HWO3	2	14.5	176.8	-0.1208955	15
HWO3	3	14.5	153.9	-1.2124378	20
HWO3	4	15	300	-0.1497512	55
HWO3	5	15	254.5	-0.219403	3
HWO2	6	7	330	1.64179104	0
HWO2	7	7	213.8	1.06368159	2
HWO2	8	7	201.1	0.95263184	1
HWO2	9	7	201.1	0.92139303	2
HWO2	10	7	143.1	0.39547264	4
PE3	11	14	283.5	0.07313433	30
PE3	12	14.5	176.8	. .	
PE3	13	14.5	165.1	. .	
PE3	14	15.5	176.8	. .	
PE3	15	15.5	176.8	. .	
PE2	16	8	194.8	-0.4412935	15
PE2	17	8	176.8	0.16766169	2
PE2	18	8	213.8	0.50099502	2
PE2	19	8	201.1	0.68402985	2
PE2	20	8	254.5	1.26616915	4
HI3	21	15.5	254.5	0.83537313	4
HI3	22	16.5	188.7	0.00004975	5
HI3	23	16.5	176.8	-2.1838308	36
HI3	24	16	188.7	0.89094527	8
HI3	25	16	213.8	0.87223881	3
HI2	26	9.5	363.1	1.55636816	2
HI2	27	9.5	227	1.08148756	2
HI2	28	9.5	188.7	0.71900498	3
HI2	29	9.5	176.8	0.85517413	1
HI2	30	9	176.8	0.659801	1
FTL3	31	17.5	213.8	0.45313433	3
FTL3	32	17.5	240.5	0.43069652	2
FTL3	33	17.5	201.1	0.95263184	1
FTL3	34	17.5	201.1	0.28835821	5
FTL3	35	17.5	176.8	0.40681592	2
FTL2	36	9.5	254.5	0.98383085	3
FTL2	37	9	254.5	1.26577841	1
FTL2	38	9.5	213.8	0.74716418	2
FTL2	39	9	176.8	0.76139303	2
FTL2	40	9	165.1	0.30462687	2

Appendix III

Accumulations of cellular-diagnostic parameters in *Porites astreoides* sampled off Broward County, Florida; Biscayne National Park and Florida Keys National Marine Sanctuary, Florida; and Little Exuma Island, Bahamas.

Site	Coral #	Hsp 60	Ubiquitin	sHsp	GRP75	Cu/Zn SOD
HWO3	1	8.29	920	2.58	15	2.69
HWO3	2	7.07	459	1.56	6.14	2.07
HWO3	3	2.57	218	0.61	7.39	1.22
HWO3	4	3.64	199	0.88	20.7	1.34
HWO3	5	8.69	975	1.67	8.14	2.25
HWO2	6	4.68	1208	1.72	4.62	2.28
HWO2	7	3.2	550	1.23	8.55	1.79
HWO2	8	3.28	1228	0.91	5.3	1.58
HWO2	9	4.63	1190	2.57	10.3	2.19
HWO2	10	4.06	1688	2.45	3.55	2.5
PE3	11	5.76	219	0.32	8.42	0.88
PE3	12	3.95	833	1.63	0.63	1.95
PE3	13	10.3	847	1.34	8.98	2.42
PE3	14	4.16	442	1.2	1.42	2.01
PE3	15	6.01	1391	3.76	5.73	3.23
PE2	16	4.25	377	1.2	2.07	1.51
PE2	17	5.79	466	0.4	12.3	1.35
PE2	18	5.12	547	2.33	2.79	3.01
PE2	19	.	.	.	.	.
PE2	20	4.74	298	0.61	7.72	1.62
HI3	21	3.75	585	1.89	2.92	2.84
HI3	22	6.16	619	2.19	2.65	2.29
HI3	23	1.89	302	0.94	1.11	1.7
HI3	24	6.92	1077	4.47	4.23	3.1
HI3	25	1.61	527	0.82	0.91	1.75
HI2	26	0.95	185	0.89	0.89	1.52
HI2	27	-2.2	68.5	0.34	0.47	0.65
HI2	28	5.02	1418	2.1	4.89	2.36
HI2	29	0.78	136	0.45	10.3	0.72
HI2	30	1.6	222	1.51	0.45	1.85
FTL3	31	3.66	556	1.94	2.27	2.09
FTL3	32	3.81	599	1.51	1.45	1.85
FTL3	33	8.46	1297	2.85	5.23	2.6
FTL3	34	8.12	522	4.75	3.45	2.17
FTL3	35	3.04	471	1.12	1.06	1.52
FTL2	36	2.76	244	1.27	1.72	1.05
FTL2	37	4.32	526	2.46	2.58	2.31
FTL2	38	1.19	1261	0.77	0.98	1.66
FTL2	39	2.93	876	1.82	1.99	2.78
FTL2	40	4.81	540	1.54	2.42	1.4
BNP	Al	2.75	321	0.96	3.51	1.51
BNP	Al	4.36	1205	1.44	2.27	3.27
FKNMS	Lo	3.01	2113	2.11	1.05	2.29
FKNMS	Lo	6.81	319	1.67	6.6	1.73

Site	Coral #	Hsp 60	Ubiquitin	sHsp	GRP75	Cu/Zn SOD
FKNMS	Mo	8.71	587	0.52	22.7	1.53
FKNMS	Mo	0.95	85.4	0.72	10.9	0.99
FKNMS	Ma	7.78	172	1.26	12.3	1.53
Bahamas	Ba	-2.6	6.84	0.34	0.54	0.13
Bahamas	Ba	-2.6	28.8	0.34	0.53	0.17

Site	Coral #	FC	CYP 2	CYP 6	cnidarian GST	MDR
HWO3	1	6.76	2.22	3.15	3.86	0.07
HWO3	2	7.42	2.8	3.07	3.37	0.07
HWO3	3	4.02	5.08	1.38	4.45	0.1
HWO3	4	5.65	0.41	3.11	1.63	0.04
HWO3	5	6.73	6.88	1.71	4.73	0.1
HWO2	6	5.02	0.99	3.01	2.66	0.06
HWO2	7	4.43	1.01	3.17	2.58	0.05
HWO2	8	3.35	1.99	1.41	3.77	0.05
HWO2	9	4.5	1.5	3.16	2.4	0.05
HWO2	10	4.53	3.81	3.02	4.37	0.05
PE3	11	4.88	0.71	2.97	2.36	0.04
PE3	12	3.78	4.31	1.71	5.29	0.05
PE3	13	6.52	3.58	1.7	5.9	0.08
PE3	14	3.72	1.05	1.94	1.64	0.12
PE3	15	10	5.76	2.2	4.25	0.2
PE2	16	6.19	1.75	2.05	1.99	0.1
PE2	17	7.84	3	1.56	3.28	0.06
PE2	18	6.9	2.67	2.21	3.48	0.13
PE2	19	.	.	.	.	.
PE2	20	6.68	1.29	1.81	2.97	0.06
HI3	21	5.11	4.41	2.2	5.51	0.15
HI3	22	5.42	3.81	2.17	3.69	0.13
HI3	23	3.56	3.18	3.02	2.99	0.07
HI3	24	7.82	5.92	1.91	3.9	0.17
HI3	25	4.37	6.03	2.51	4.7	0.13
HI2	26	2.89	1.12	1.51	1.1	0.08
HI2	27	0.87	0.29	0.96	0.46	0.03
HI2	28	5.7	1.78	3.17	2.41	0.05
HI2	29	2.29	0.42	1.32	1.08	0.02
HI2	30	3.44	2.05	1.28	3.94	0.03
FTL3	31	5.65	1.41	1.9	1.97	0.12
FTL3	32	4.96	0.62	3.68	2.98	0.09
FTL3	33	9.51	7.24	3.36	4.68	0.15
FTL3	34	5.3	4.18	1.48	3.03	0.14
FTL3	35	3.94	0.46	3.36	2.31	0.09
FTL2	36	4.6	0.91	1.35	1.84	0.08
FTL2	37	6.17	1.48	1.96	2.55	0.13
FTL2	38	2.91	1	1.91	1.73	0.09
FTL2	39	5.47	1.32	2.71	2.84	0.15
FTL2	40	6.93	0.99	1.7	2.59	0.12
BNP	Al	2.99	0.57	1.88	2.63	0.07
BNP	Al	6.42	3	1.95	3.07	0.15
FKNMS	Lo	4.09	1.32	2.82	4.18	0.07
FKNMS	Lo	5.58	5.7	1.88	4.62	0.06
FKNMS	Mo	5.26	2.56	2.67	3.53	0.05
FKNMS	Mo	1.78	0.93	1.35	2.09	0.03
FKNMS	Ma	3.53	0.81	1.4	2.49	0.06
Bahamas	Ba	0.09	0.32	0	0.21	0
Bahamas	Ba	0.35	0.48	0.1	0.33	0

Appendix IV

Accumulations of cellular-diagnostic parameters in *Porites astreoides* during the laboratory experiment.

Sample	Water	Depth	Colony	Hsp 60	Ubiquitin	sHsp	GRP 75
10J-51	PE	Shallow	J	1.88	1959	0.5	13.1
11J-56	ASW	Shallow	J	2.24	1938	0.26	10.2
12C-21	PE	Deep	C	4.32	2767	0.21	13.9
13C-16	ASW	Deep	C	3.12	2925	0.45	7.98
14E-27	ASW	Shallow	E	1.76	403	0.28	4.53
14E-31	ASW	Shallow	E	2.52	645	0.23	6.68
15A-5	PE	Deep	A	6.77	1431	0.24	14.5
16J-52	ASW	Shallow	J	0.83	1884	0.26	4.66
17E-26	PE	Shallow	E	2.05	539	0.19	6.72
18C-18	PE	Deep	C	3.3	2096	0.28	13.8
19E-34	ASW	Shallow	E	2.25	271	0.2	5.87
20E-25	PE	Shallow	E	3.1	441	0.18	7.27
20E-35	PE	Shallow	E	1.19	115	0.35	3.99
21A-2	ASW	Deep	A	7.08	1144	0.35	17
23J-59	PE	Shallow	J	2.17	3235	0.39	17.9
24J-50	PE	Shallow	J	1.81	2956	0.25	15.7
2C-24	ASW	Deep	C	3.09	2872	0.18	8.64
3A-4	PE	Deep	A	5.5	1186	0.19	13
4E-32	ASW	Shallow	E	2.75	879	0.21	9.61
5C-20	ASW	Deep	C	3.32	2912	0.18	12.1
6A-7	PE	Deep	A	7.7	1482	0.2	21.8
7C-19	PE	Deep	C	2.25	1485	0.42	9.01
8J-49	ASW	Shallow	J	2.01	2088	0.18	9.55
9A-1	ASW	Deep	A	6.53	988	1.49	16.3

Sample	Cu/Zn SOD	FC	CYP P450 6 class	cnid GST	MDR
10J-51	2.61	5.81	4.2	5.46	0.04
11J-56	2.31	5.48	1.58	4.32	0.06
12C-21	4.19	4.48	1.83	6.81	0.08
13C-16	3.39	4.12	0.9	6.13	0.05
14E-27	1.66	3.37	0.93	2.94	0.03
14E-31	2.5	4.59	2.81	4.19	0.05
15A-5	6.17	5.61	3.41	10.5	0.13
16J-52	1.37	3.21	0.98	2.47	0.02
17E-26	2.25	3.83	1.19	3.92	0.04
18C-18	2.75	5.55	1.06	5.68	0.07
19E-34	2.34	4.07	1.36	3.35	0.04
20E-25	2.36	5.12	4.62	5.81	0.06
20E-35	1.79	2.62	0.92	2.79	0.02
21A-2	6.09	7.34	5.3	10.3	0.2
23J-59	3.00	6.57	5.79	5.1	0.05
24J-50	2.88	4.92	5.07	6.31	0.03
2C-24	2.38	3.78	0.85	4.72	0.05
3A-4	5.23	4.82	3.15	10.1	0.1
4E-32	3.27	4.76	2.79	5.47	0.06
5C-20	3.53	5.48	1.17	5.92	0.08
6A-7	7.37	7.95	5.69	11.8	0.18
7C-19	2.12	4.45	0.81	3.49	0.04
8J-49	2.76	4.54	3.57	4.7	0.04
9A-1	6.07	7.21	3.95	11.4	0.14