Final Report
Biomarker Study to Identify and Trace Coral Reef Contaminants

Southeast Florida Coral Reef Initiative
Land-based Sources of Pollution
Local Action Strategy Project 5

Southeast Florida Coral Reef Initiative
Acting above to protect what's below.

Biomarker Study to Identify and Trace Coral Reef Contaminants

Final Report

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EXECUTIVE SUMMARY

Southeast Florida's coral reefs are part of the world's third largest coral reef ecosystem. Divers rate them among the Top 10 sites worldwide for viewing marine life, and annual reef-related expenditures for fishing, diving and boating contribute $6.3 billion in income and sales and support more than 71,000 jobs. The asset value of natural reefs in Palm Beach, Broward, Miami-Dade and Monroe counties was $8.5 billion; in Wall Street terms, Florida's coral reefs are a mid-cap business.

Managing southeast Florida's coral reefs is challenging because they lie just offshore of the largest metropolis in Florida. Land-based sources of pollution ranging from secondary treated wastewater to storm water, fertilizers, sedimentation and other stressors can harm these reefs. In recent decades, Caribbean reefs declined enough that two dominant coral species (staghorn and elkhorn corals) were federally listed as threatened species. Our project explored linkages between contaminant loads in water, sediment, and coral tissues, and coral reef condition. The objective was to determine the chain of causality between land-based pollutants, responses of individual reef-building corals, and the health of coral reef communities. It included tests of causal relationships that used established experimental protocols, including fertilization assays with sea urchins, settling experiments with coral larvae, and a reciprocal transplant experiment.

Traditional monitoring methods identified increased percentages of bare ocean floor near two Broward County ocean outfalls. A sentinel coral species (*Porites astreoides*) was absent from one station at both the Port Everglades channel and Hillsborough Inlet. A new method we developed to evaluate the balance between algal growth and herbivory revealed increased cover by red algae near the Hollywood ocean outfall. *Porites astreoides* continued to have difficulty repairing experimentally-induced lesions at 75% of deep-water stations, and was temporarily buried in sediment at one station. At three other stations, we found pore water contained sufficient freshwater to kill sea urchin embryos. Other researchers isolated human enteric pathogens from some of these stations, which established a linkage to human fecal contamination.

Laboratory tests showed reduced ability of coral larvae to settle due to sedimentation, and on substrates conditioned at the deep water Port Everglades station. Sea urchin fertilization and development tests had variable results, with signs of impairment from all stations except Hillsborough Inlet. Biomarker assays showed stress levels varied significantly with the exact combination of station and year. One pattern was consistent: frequencies of DNA damage and concentrations of the antioxidant enzymes catalase and Cu/ZN SOD were

elevated at midwater stations near Port Everglades and Hillsborough Inlet. This pattern suggests chronic exposure to these point sources of pollution stressed corals, which mounted antioxidant defenses but still sustained some DNA damage. However, damage levels and antioxidant concentrations were lower than those off St. John (U. S. Virgin Islands), where mass coral mortality events occurred during the same time span. A reciprocal transplant experiment is underway to test the relative importance of environmental and genetic factors on growth of *P. astreoides* at the other three deep water stations off Broward County. After one year of deployment, colonies increased in surface area by 15%, with no significant variation attributable to stations of origin or transplantation, or their interaction.

Overall, the results of this project are consistent with our previous study and a recent assessment of human fecal contamination. We found evidence of negative effects of shipping channels and ocean outfalls on coral reefs at multiple biological levels, ranging from DNA and gametes to populations and the whole ecosystem. No station was exempt from some negative outcome, which reinforces the need to use more distant, less impacted sites (e.g., Bahamas) as reference stations. Some effects, such as burial by sediments, occurred on very small scales both spatially and temporally. The overall pattern for coral reefs off Broward County is consistent with intermittent chronic stress rather than acute stress, and portends continued slow, sporadic declines of the southeast Florida coral reef. However, this outcome can be reversed by effective management, including closing the ocean outfalls on (or before) schedule, reducing run-off onto the reef, and limiting discharge of agricultural, industrial and urban wastes from canals. To accomplish this, we recommend adopting a cohesive research and management protocol—similar to that employed by the Amphibian Research and Monitoring Initiative—that includes both monitoring and testing, and expanding partnerships with agriculture, municipalities, and tourism. We strongly support co-locating scientific studies, monitoring environmental conditions at the same place and time as biological responses, and using an adaptive, problem-solving approach to managing southeast Florida's valuable coral reefs.

1 INTRODUCTION

The southeast Florida coast includes the northernmost extension of the world's 3rd largest coral reef ecosystem, which stretches nearly 500 km from St. Lucie Inlet in Martin County to the Florida Keys and the Dry Tortugas. Dive magazines consistently rank southeast Florida reefs among the Top 10 sites worldwide for viewing large fishes and other marine life (SCUBA Diving magazine, 2008). These reefs support a vigorous commercial and recreational fishery, with landings of fishes and invertebrates (mainly spiny lobster, stone crab claws, blue crabs, and shrimp) exceeding 20 million pounds a year (Moberg and Folke, 1999). Fishing, diving and boating provides tremendous income for coastal communities in southeastern Florida; annual reef-related expenditures contribute $6.3 billion in income and sales and support more than 71,000 jobs (Collier et al., 2008). The asset value of natural reefs in Palm Beach, Broward, Miami-Dade and Monroe counties of southeast Florida was $8.5 billion (Johns et al., 2001), which exceeds the gross domestic product of 72 countries, including The Bahamas and Belize (U. S. Central Intelligence Agency, 2009).

This rich natural and economic resource lies just offshore of Florida's most developed coastline and three of the largest metropolitan areas in the U.S.: Miami-Dade (#8), Broward (#15) and Palm Beach (#29) counties. Most of their urban development is within 20 km of the coast, and increasing urbanization and land-use changes have been identified as major threats to southeast Florida's reefs. Wastewater pollution, sedimentation, eutrophication and altered water flows that accompany coastal development and shipping operations also are threats to reef health (Burke et al., 2004).

The goal of our project was to identify and trace stressors affecting coral condition and to use experiments to test causal relationships. Land-based sources of pollution are a critical factor affecting the condition of coral reefs off the southeastern Florida coast. In 2006, a pilot study (Fauth et al., 2006; Dustan et al., 2008) used a cellular-diagnostic system to link physiologically-stressed corals with inlets and ocean outfalls. Cellular-diagnostics assesses organismal condition using a suite of enzymes and other molecules, in a manner similar to biomedical tests (Downs, 2005). Cellular-diagnostic parameters were elevated at all eight Broward County stations compared to mustard hill coral (*Porites astreoides*) colonies sampled in the Commonwealth of The Bahamas. Two stations near the City of Hollywood's ocean outfall were characterized by high levels of GRP 75 (mortalin), which was associated with coral tissue loss. Colonies at these sites also had high levels of ubiquitin, which tags damaged proteins for degradation. Combined, these results suggested that coral nutrition was altered by treated wastewater discharge and resulted in above-normal protein turnover (Fauth et al., 2006; Dustan et al., 2008). Corals at three offshore sites also had high levels of

heat shock protein 60, cytochrome P-450 II class enzymes and multi-drug resistance protein, which are consistent with oxidative damage caused by exposure to xenobiotics: chemical compounds foreign to living organisms. Together, these results demonstrated that offshore corals at the biomonitoring site and off Port Everglades and Hillsborough Inlet were exposed to anthropogenic contaminants. Regeneration of coral biopsies was negatively correlated with elevated levels of cytochrome P-450 II class enzymes, which suggests that defending against xenobiotics had a metabolic cost: coral colonies could not repair damaged tissue as quickly. Alleviating such chronic stresses caused by land-based sources of pollution is essential for maintaining the economically-valuable coral reefs ($6.3 billion; Johns et al., 2001) off the southeast Florida coast.

The current project expands our earlier work (Fauth et al., 2006; Dustan et al., 2008) by incorporating screening tests for acute toxicity with exploratory contaminant analysis of pore water, sediment, and coral tissues, to identify potential sources of stress (Fig. 1). It also expands the coral monitoring protocol by sampling at two locations per station and evaluating coral recruitment, which is essential for long-term survival of coral reefs. This approach provides more than correlations between potential stressors and coral responses; it includes linkages between contaminant loads in water, sediment, and coral tissues, and tests for causal relationships using established experimental protocols, including acute toxicity tests with brine shrimp and fertilization assays with sea urchins. The overall project objective is to identify the chain of causality between land-based pollutants, responses of individual reef-building corals, and the health of coral reef communities within the southeast Florida watershed (Fig. 2).

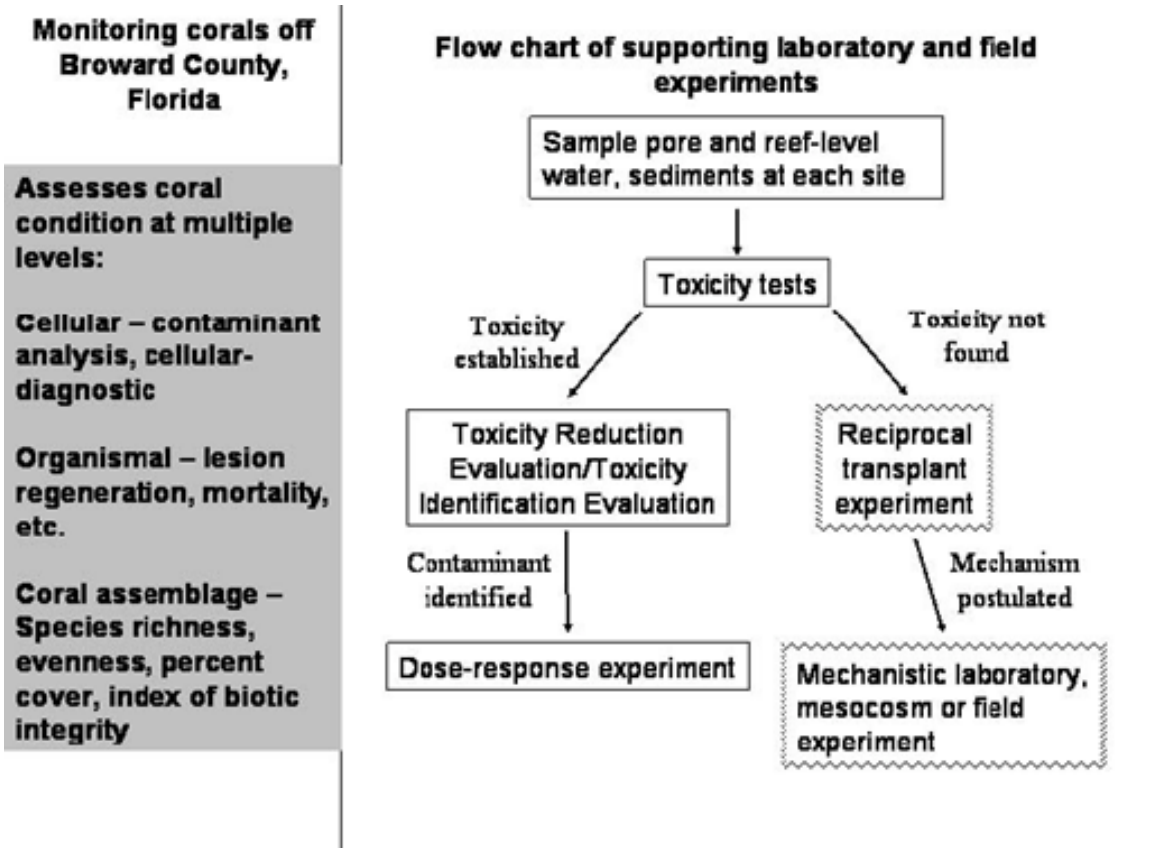


Figure 1. Summary of the research plan, which was designed to identify linkages between coral condition and land-based sources of pollution.

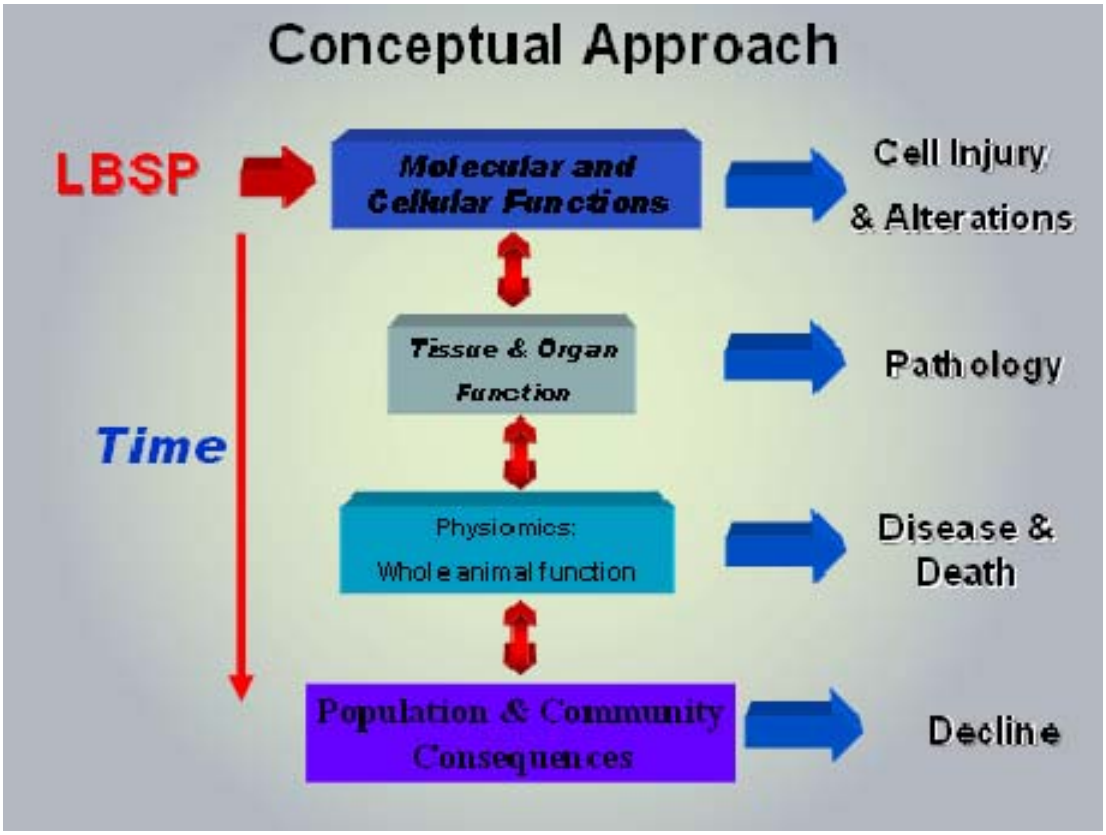


Figure 2. Land-based sources of pollution (LBSP) move through the air and water (horizontal arrow, top) and enter living cells, where they first affect molecular and cellular functions. If cellular defenses are overwhelmed, higher levels of the biological hierarchy can be affected, leading to decreased health, disease, and ultimately the decline of coral reefs.

2 METHODS

2.1 Study sites

Our sampling methods were identical to those of our pilot study (Fauth et al., 2006) with one exception: at the request of the Florida Department of Environmental Protection, we quantified within-station variation by sampling corals at each end of a 25 m transect within each station (Fig 3). We sampled at the same four paired inshore-offshore stations as before; these were selected based on proximity to different potential sources of land-based pollution: a treated wastewater discharge outfall (Hollywood Outfall), an inlet (Port Everglades), a station with both a treated wastewater discharge outlet and an inlet in close proximity (Hillsborough Inlet), and a biomonitoring control station (Fig 4).

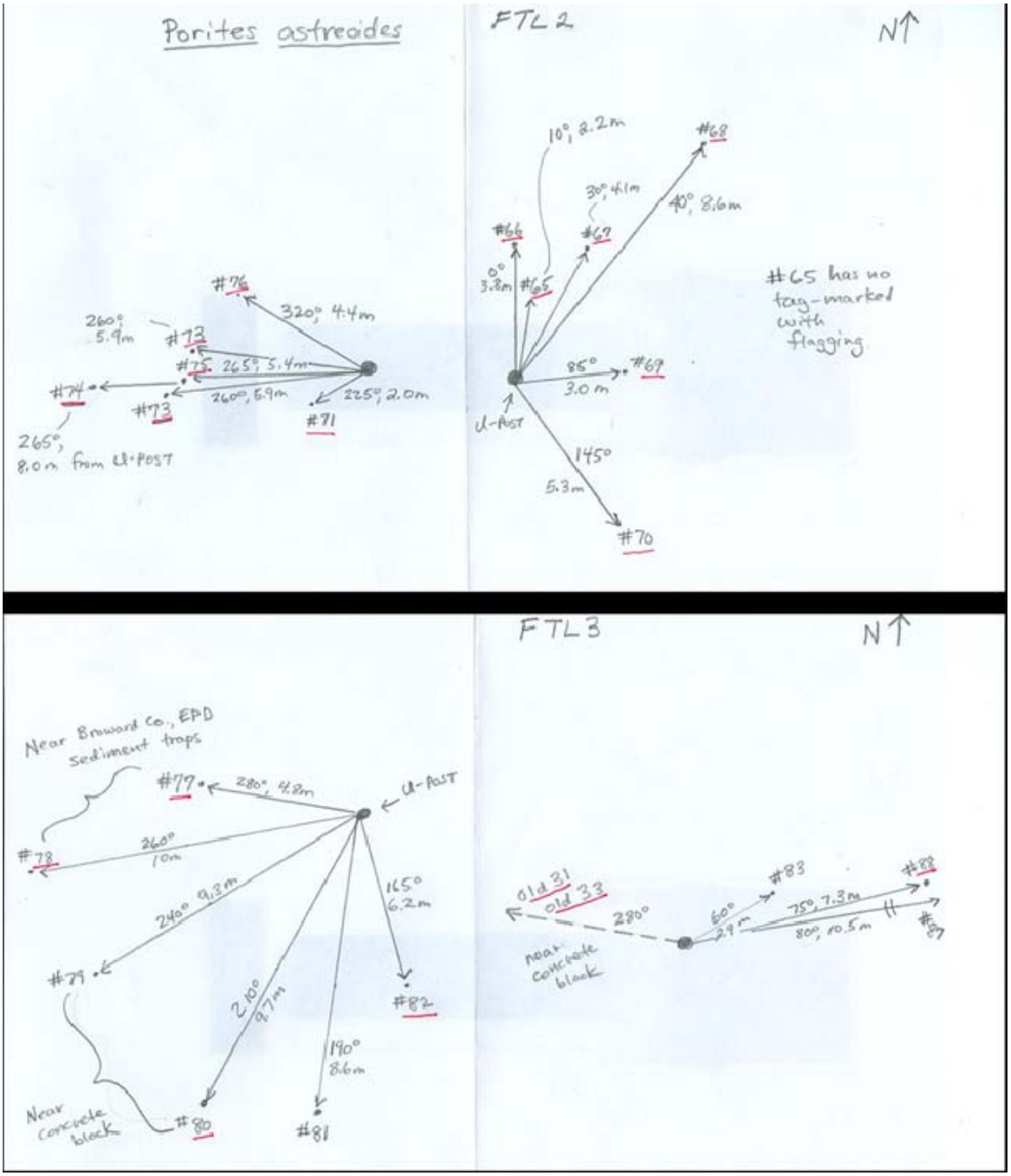


Figure 3. Scanned image of the map used by divers to locate corals at Broward County Environmental Protection Department's control stations (C2, top; C3, bottom). The two U-posts (solid circles) were 25 m apart and held a sediment trap and temperature logger. Vectors give compass bearings and distances of tagged colonies of *Porites astreoides* from the nearest U-post. Two colonies at the C3 station (Old 31 and Old 33) were included in the previous pilot study (Fauth et al. 2006). Video transects were conducted along the 25 m line between the two U-posts.

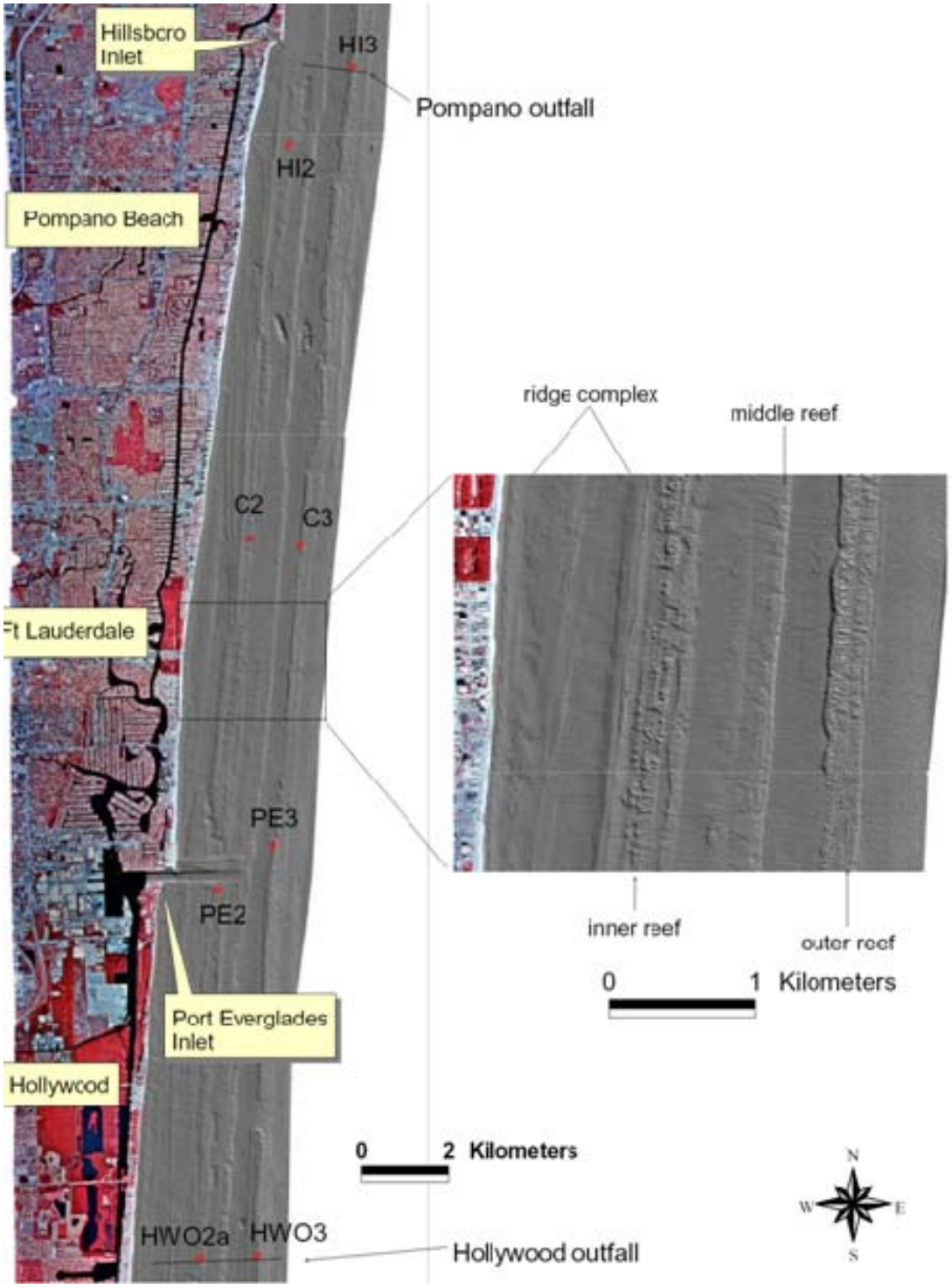


Figure 4. Location of the four paired sampling stations showing their proximity to urban areas and potential land-based sources of pollution off the coast of Broward County, Florida, USA. Reproduced from Fauth et al. (2006).

The two ocean outfalls discharge equivalent volumes of treated secondary wastewater and other contaminants but the Port Everglades channel is much larger than Hillsborough Inlet (Futch et al., 2011 and references therein). Our deepwater stations near ocean outfalls were <200 m from their surface boils and stations near shipping channels and inlets straddled them as closely as possible. This sampling design permitted variation in coral reef responses to be partitioned between depths, between proximity to outfalls and inlets, and—because of the within-station replication—all their possible interactions. Other research and monitoring groups are co-locating studies at these sites (e.g., human fecal contamination: Futch et al., 2011).

2.2 Ecosystem Assessment

The geomorphology of this region was characterized recently by Banks et al. (2007) and its community composition by Sathe et al. (2009). To assess conditions at our stations, we used the Atlantic & Gulf Rapid Reef Assessment (AGRRA), VitaReef, Point Count, and quadrat sampling methods to estimate coral species richness, evenness, Shannon-Weiner diversity, apparent species richness (Jost 2006), percent cover, size-structure, recruitment, and the distribution of bleaching and disease. To assess coral reef community structure, we examined digital video imagery collected along both sides of the 25 m transect at each paired stations. 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, poriferans, macroalgae, and members of the cyanobacteria genus *Lyngbya*.

2.3 Coral regeneration rates

On July 31-August 1, 2007, we located 8–12 colonies of the mustard hill coral (*Porites astreoides*) at each station, distributed as evenly as possible at opposite ends of the transect. We identified colonies using uniquely numbered cattle ear tags, which we secured to nearby substrates using plastic cable ties. We returned to all stations multiple times to re-assess coral condition. We visibly inspected coral colonies and re-photographed individual biopsy lesions, digitized the images, and estimate coral regeneration rates and total colony percent cover, as described in Fauth et al. (2006) and Fisher et al. (2007).

2.4 Cellular diagnostics

We used methods similar to those reported previously (Fauth et al., 2006; Dustan et al., 2008). Briefly, at each station we used a steel punch to remove a 1.5 cm diameter biopsy from 3-6 colonies of the reef-building coral *P. astreoides* at each end of our 25 m transect. Tissue samples were placed into individual labeled

containers underwater, returned to the surface, and transported to the laboratory in a liquid nitrogen cryoshipper. Tissue samples were stored at -80° C for cellular-diagnostic systems (CDS) analysis.

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 (catalog #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).

We ground frozen coral samples to a powder using a liquid nitrogen-chilled ceramic pestle and mortar. We placed frozen tissue samples (~10 mg) into 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 µM a-amino-caproic acid, and 1 µg/100 ul pepstatin A. We heated samples 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. We then centrifuged samples for 10 min at 10,000x g. We transferred supernatant free of the lipid/glycoprotein mucilage matrix to a new tube, centrifuged for 5 min at 10,000x g and again transferred free supernatant to a new tube and subjected it to a protein concentration assay using methods adapted from Ghosh et al. (1988).

To ensure equal sample loading, we loaded 20 µg of total soluble protein (TSP) of samples onto a 12.5% SDS-PAGE gel (16 cm), ran the gel until the bromophenol blue dye front was near the bottom, stained it 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 µg TSP of coral supernatant was loaded onto a 20-, 16-, or 8-cm SDS-polyacrylamide gel with various concentrations of bis/acrylamide. We blotted gels onto PVDF membrane using a wet transfer system. We blocked membrane in 7% non-fat dry milk and incubated with primary antibody for 1 h. We washed blots 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. We then washed blots four times in TBS and developed them using a chemiluminescent reporter system.

Once validated, we optimized antibodies and samples for ELISA using an 8 x 6 x 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). We used A Bio-Tek 404 plate washer in conducting the ELISAs using a 96-well micro-titer plate format. We developed antibodies using a luminol-based chemiluminescent solution and documented them using a Bio-Tek fluorescent/luminescent microplate reader.

We assayed samples with Haereticus Environmental Laboratory's antibodies against catalase, copper-zinc superoxide dismutase (Cu/Zn SOD), and ubiquitin. The biological significance of each cellular-diagnostic parameter (biomarker) is summarized in Table 1. Samples were assayed in triplicate. We plated an eight-point calibrant curve using a calibrant relevant to each antibody in triplicate for each plate.

For the DNA AP site assay, we obtained sample DNA using the GetPur DNA Purification kit (Dojindo Molecular Technologies, Gaithersburg, MD) with the minor modifications described in Downs et al. (2011). Briefly, we placed 50–60 mg of frozen (-80˚C) ground coral tissue powder into a 1.5 ml tube containing 400 µl lysis buffer and approximately 10 mg polyvinylpolypyrrolidone (PVPP, Sigma) to remove any polyphenolic compounds associated with the sample. We vortexed samples for 5 s, added 10 µl of proteinase K solution, and vortexed the samples again. We heated this mixture for 10 min at 65°C, removed fromheat and cooled for 2 min. We then added a 2 µl aliquot of RNase solution, vortexed tubes quickly and incubated them at room temperature for 2 min. We added 80 µl of Solutions I and II supplied with the kit, vortexing tubes after each addition. We then centrifuged samples at 14,000 rcf at 4°C for 10 min, aspirated the supernatant and placed it into a clean tube. We added an equal volume of 100%

ethanol (Pharmco-Aaper, Brookfield, CT) vortexed the samples, and centrifuged again as above. We decanted the supernatant and washed the nucleic acid pellets with 1.0 ml of 70% ethanol. After a final centrifugation (14,000 rcf, 5 min), we decanted the liquid and dried pellets in a speedvac. We resuspended sample DNA in 100 µl of 10 mM Tris-1 mM EDTA (TE), pH 8.0 and placed at 4°C overnight.

We determined DNA concentration using the Quant-iT DNA HS assay kit (Molecular Probes, Eugene, OR), with recovery between 20 and 100 pg/ml. We used a DNA Damage Quantification kit (Dojindo Molecular Technologies, Gaithersburg, MD) to determine the number of aldehyde reactive abasic sites in eachnucleic acid sample with a substrate reporter substitution to increase assay sensitivity. We placed 10 µl of a 10 µg/ml solution in a 0.5 ml tube and added 5 µl of aldehyde reactive probe (ARP) solution. We then used the manufacturer's protocol for the remainder of the labeling reaction. We diluted samples (90 µl in 310 µl TE) and pipetted 60 µl 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), we added 100 µl of a 50:50 mixture of luminol:oxidizer from the Western Lightning Chemiluminescent Reagent Plus kit (Perkin-Elmer, Waltham, MA) to each well, replacing the colorimetric substrate supplied in the kit. We read the plate 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 that exceeded the range of the kit standards, we made dilutions in the TE solution, relabeled samples with ARP, and re-evaluated them. Intra-replicate variation was <8% for all but one sample (13%), with most samples <6%.

2.5 Temperature and sedimentation rate

We deployed HOBO H8 series temperature loggers (Onset Corporation, Bourne, MA; (accuracy: ± 0.7° C at 21° C) in submersible cases to continuously record ocean temperatures at 1 h intervals. One logger was attached to the U-post at the opposite ends of each station (Fig. 3).

We collected settled suspended sediments by attaching a 5.1 cm diameter x 60 cm long polyvinyl chloride (PVC) pipe to every U-post to serve as a sediment trap. Traps were capped underwater, returned to the surface, kept cool with ice, and returned to the laboratory. Sediment traps were maintained frozen until analysis. We then thawed the contents, decanted excess water, and filtered out large items using a 0.5 cm sieve. Sediment and remaining water were collected in previously-weighed beakers and dried in an oven at 60° C. The beaker was re-weighed to determine sediment mass (by difference from the empty beaker) and sediment was transferred into labeled whirl-pak bags and stored at room

temperature. We later fractioned sediment using sieves (75, 125, 250 and 500 µm; plus 1, 2, 3.35 and 4 mm) to assess size distributions among stations.

Sediment traps were deployed for weeks to months at a time and were colonized by algae, bryozoans, molluscs and other organisms. These were grazed, presumably mainly by fishes because sea urchins were uncommon at these stations. We quantified the balance of fouling versus grazing by photographing sediment traps, superimposing a set of 20, randomly distributed points on the image, and scoring them as overlying bare PVC, barnacles, bryozoans, worm tubes, red algae, green algae, or scrape (=bite marks). A point could overlay more than one category and we summarized the percentage of each cover type per trap (Fig. 5).

Table 1. Cellular-diagnostic parameters and their biological significance.

Parameter	Interpretation
Catalase	Catalase is an antioxidant enzyme found in all plants and animals, and catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen (Gaetani at al. 1996). Catalase acts in concert with superoxide dismutases to eliminate reactive oxygen species that damage the coral-algae symbiotic system.
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).
DNA AP Site	DNA interacting with reactive oxygen species (ROS) is a major cause of DNA damage, particularly 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).
Ubiquitin	Ubiquitin is a 76-residue protein found in most phyla and marks proteins for rapid degradation. 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.

Figure 5. Sediment trap recovered from PE3 in June, 2008, showing heavy colonization by marine organisms. Scrape marks from grazers are evident toward the trap's top (right side of photo). The circumferential bands of bare PVC (above the ruler) were covered by plastic cable ties that secured the trap to its U-post.

2.6 Contaminant analyses

To screen for contaminants potentially responsible for coral degradation, we sampled and stored pore and reef-level (i.e., at the depth of coral colonies) water, sediments, and coral tissue at each station. At each station, we collected three pore water samples by placing a labeled, acetone-cleaned, 60 mL syringe into the substrate, withdrawing the plunger to collect pore water, capping the end with acetone-cleaned aluminum foil, and transported the syringes to the surface. On deck, syringes were placed into individual plastic bags and stored on dry ice. We collected 2–3 reef-level and surface-water samples from each station in individually labeled, acetone-cleaned, 1 L, collapsible LDPE cubitainers (Hedwin Corporation, Baltimore, MD). On June 12, 2008, we also collected surface-water samples along a Hollywood outfall transect, including samples from the surface boil, and midway between HWO3 and HWO2. Both at depth and at the surface, cubitainers were expanded by pulling on their flexible sides, allowing seawater to enter, then sealed with their screw-on cap. On deck, cubitainers were stored on ice. In the laboratory, all water samples were refrigerated until they were used in ecotoxicology experiments. All pore-water samples were consumed during these experiments but surplus reef- and surface-water samples were stored in freezers at -20° C.

We collected 2-3 sediment samples from each station in individually-labeled, 250 mL short, clear or amber wide-mouth jars (Eagle Pitcher or Scientific Products), which are certified to comply with or to be lower than the EPA detection limits for metals, salts, semivolatiles, volatiles, and pesticides/PCBs. Before each dive, jar lids were loosened to allow air to escape and prevent the jar from exploding at depth. A diver then used the jar to scoop the top 2 cm of sediment, capped the jar, and returned it to the surface. On deck, sediment samples were placed on ice for transportation to the laboratory, where they were stored in a -20° C freezer.

We sampled 1-2 coral colonies at each paired site within a station for contaminants, using the same procedures described previously for cellular-diagnostic analysis. However, tissue samples were placed into individually-labeled, clean Teflon vials instead of camera film cases. Coral tissue samples were stored at -80° C until analysis. Sediments and coral tissue samples can be assayed for common contaminants (e.g., pesticides, herbicides, polyaromatic hydrocarbons, industrial chemicals) using GC-MS and other techniques.

2.7 Larval settling experiments with coral planulae

We performed two experiments at Nova Southeastern University's National Coral Research Institute to determine if contaminants within sediment or colonizing substrates affected settlement of larval *P. astreoides*. To obtain coral planulae, we collected 10-15 mature colonies of *P. astreoides*, transported them to the Nova Southeastern University Oceanographic Center and placed them in an outdoor seawater system. Each colony was placed in a larval collection bucket within the seawater system and supplied with a continuous flow of water. Larvae were collected over two (experiment 2) or three (experiment 1) consecutive mornings and distributed equally among experimental aquaria (i.e., we added an equal number of larvae from a single colony to each aquarium). In the first experiment, we added a total of 60 larvae per tank over 3 d, with larvae obtained from three colonies of *P. astreoides*. We added 33 larvae on the first day, 16 on the second, and 11 on the third and last day. In the second experiment, we added a total of 45 larvae obtained from two parent colonies. Forty were added the first day, and five were added the second day.

We conducted both experiments in separate 9.5 L, closed-system aquaria randomly distributed within a water bath (Fig. 6). Aquaria were maintained under the same temperature, salinity, and circulation conditions. Aquarium water consisted of filtered and treated seawater from a saltwater well. Water circulation was provided during portions of the first experiment by Aquaclear Mini™ power filters. Light was provided by metal halide lamps (2 x 175 W, photoperiod 12:12 h). We monitored salinity using a refractometer and maintained it at approximately 35 ppt. Temperature was maintained at approximately 26°C.

For the first experiment, we turned off water circulation pumps before adding larvae and performed twice daily, 1 L water changes to maintain water quality. For the second experiment, once daily 75% water changes were performed. Once no larvae were observed in the water column (several days after their addition), we turned pumps back on in the first experiment and added a continuous stream of water into the aquaria in the second experiment to maintain water movement and water quality. In the first experiment, we used a microscope to check the

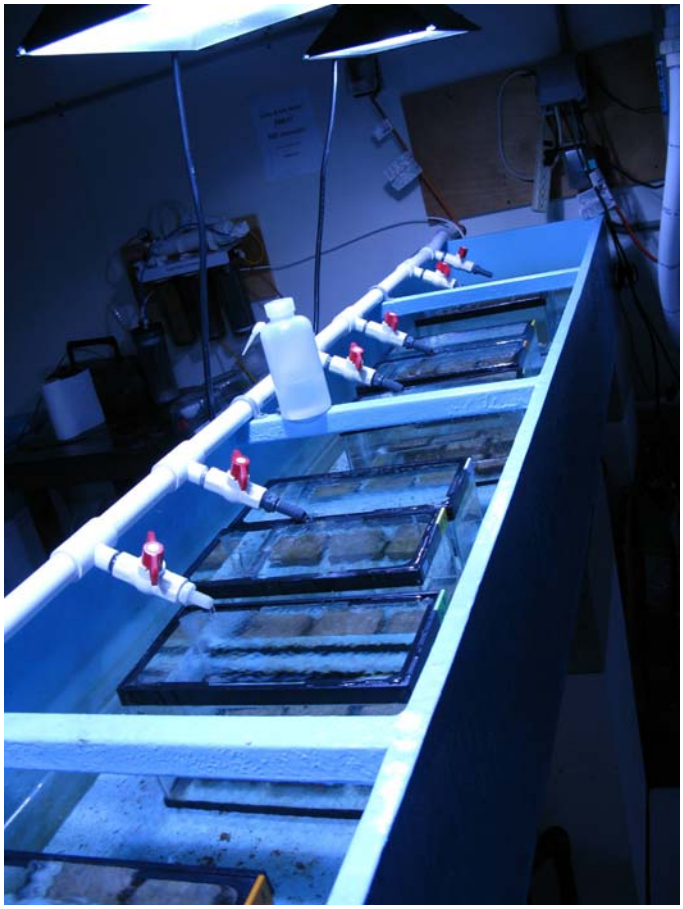


Figure 6. The 2011 experiment at Nova Southeastern University's Oceanographic Center, showing aquaria within the water bath. Each aquarium had plates from a single site.

terracotta plates for settled coral 23 d after larvae were added. For the second experiment, we used a flashlight with a blue LED that excited green fluorescent proteins in the *P. astreoides* spat to aid with detection. Cement plates were checked 11-13 d after larvae were added to the tanks.

We conducted the first experiment during the spring 2009 spawning season to determine whether natural substrates affected coral settlement. Divers collected 3 L of sediment and several rocks from two offshore sites: Control Station 3 (C3) and Hillsboro Inlet 3 (HI3). We collected sediment in 1 L jars, and wrapped rocks in wet paper and transported them in plastic bags. Sediment and rocks from each site were placed into three replicate aquaria per treatment, with an additional control consisting of 1 L of clean, dry CaribSea Aragonite Sand. We placed three terracotta settlement plates into each tank and conditioned them for 3 d. At the end of the conditioning period, we removed rocks to limit potential settlement surfaces to the standardized terracotta plates.

We conducted the second experiment during the spring 2011 spawning season to determine whether benthic organisms that recruited onto settlement plates

affected larval establishment. We deployed settlement plates into the three stations used for the reciprocal transplant experiment (C3, PE3 and HWO3) on April 1 and collected them May 24. We conditioned an additional set of plates in NCRI's seawater system nursery, which is supplied with saltwater from a well and treated with ozone, a protein skimmer, and Seaclear to remove phosphate (lanthanum chloride precipitates phosphate as lanthanum phosphate which is removed with a 1 micron filter). We replicated each of these four treatment three times, in a total of twelve independent aquaria, each containing three tiles from a given station (Fig. 7).

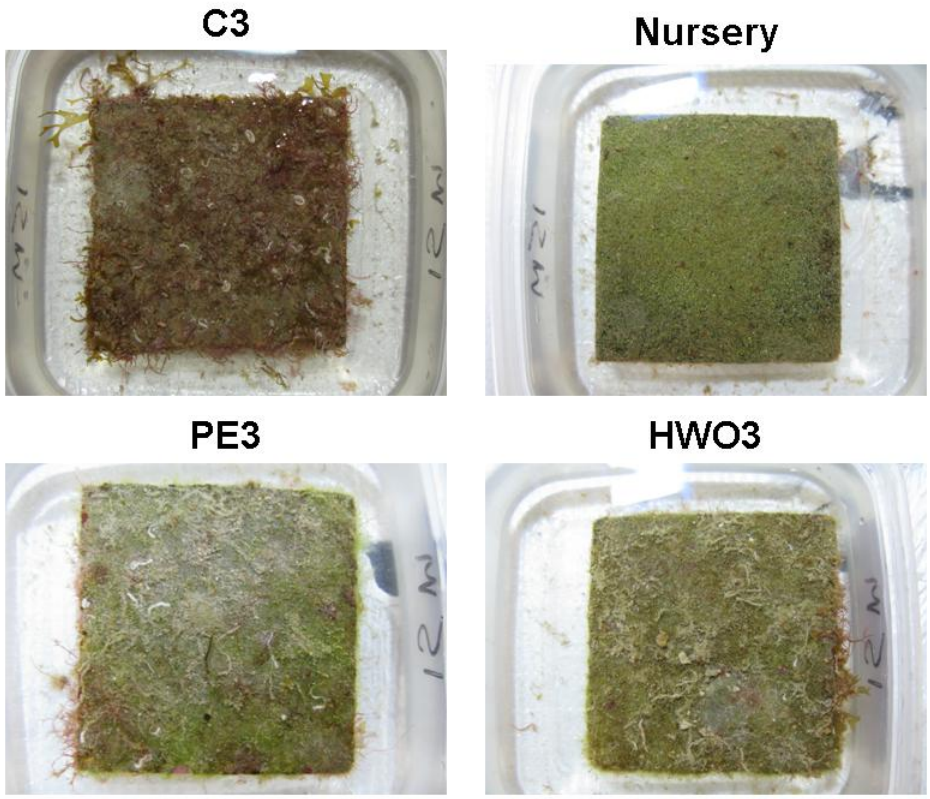


Figure 7. Representative plates from each of the four stations used in the 2011 larval settlement experiment.

2.8 Toxicity Identification Experiments

We tested whether water- or sediment-borne constituents had an acute toxic effect on coral reef organisms using two methods: 1) An acute toxicity test with brine shrimp (*Artemia salina*), which is a relatively stress-tolerant species, and 2) a sea urchin fertilization assay using *Lytechinus variegatus* or *Arbacia purpurea*. Both urchin species are less stress-tolerant than brine shrimp.

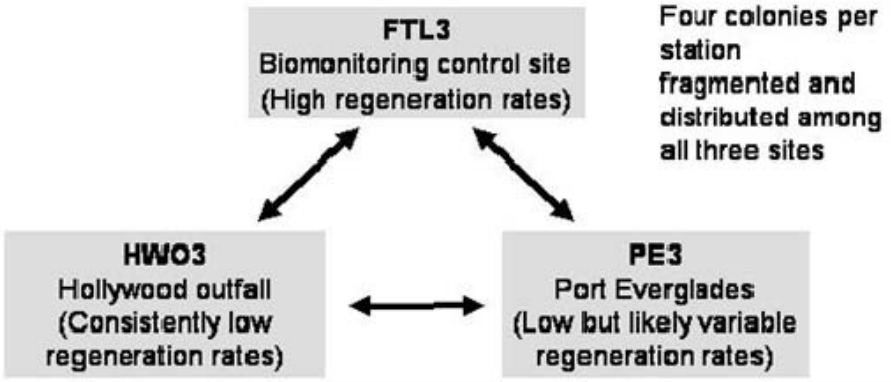
We improved the brine shrimp acute toxicity test of Carballo et al. (2002) by counting the initial number of unencysted eggs aliquoted into the test chamber, and determining the percentage reaching the nauplius stage. This was more precise than comparing the number of nauplii in each treatment with the number in the control, which assumes that the initial number of nauplii was constant across all treatments. Tests were conducted on unfiltered reef-level water and on pore water filtered through a 13 mm sterile, 0.45 µm PVDF filter. Artificial seawater was used as the control.

We extended the Sea Urchin, *Arbacia punctulata*, Fertilization Test Method 1008.0 (U. S. Environmental Protection Agency) by rearing embryos to the pluteus stage. Tests were conducted on unfiltered reef-level water and on pore water filtered through a 13 mm sterile, 0.45 µm PVDF filter. Artificial seawater optimized for sea urchin development was used as the control, and contained (per L): 28.32 g NaCL, 0.77 g KCL, 5.41 g $MgCl_2 \cdot 6H_20$, 6.98 g $MgSO_4 \cdot 7H_20$), 0.2 g $NaHCO_3$ and 1.56 g $CaCl_2$ dihydrate. The pH was brought to 8.2 using NaOH and salinity was maintained between 34-36 ppt.

2.9 Reciprocal transplant experiment

Because acute toxicity was not detected, we did not screen for potential contaminants. Instead, we initiated a reciprocal transplant experiment (Fig. 1) using *P. astreoides* in March, 2011. Reciprocal transplants are a powerful experimental method for evaluating the effect of local environmental conditions. Our design (Fig. 8) concentrated on three offshore stations that had very different coral regeneration rates: the biomonitoring control site (C3), Port Everglades (PE3), and the Hollywood outfall (HWO 3). It also exploited the clonal nature of corals to assess the relative contributions of ecotypic variation, environments, and their interactions on coral growth rates and CDS parameters. Briefly, we collected 4-5 small colonies of *P. astreoides* from each of these three sites and returned them to the laboratory in coolers filled with seawater from their site. We then used a tile saw to cut each colony into four small explants. Because P. *astreoides* grows by fission, these explants constitute genetic clones. We randomly selected one explant and subdivided it, flash- freezing one half for CDS and contaminant analysis and preserving the remainder in Z-fix for histological examination. These samples established the baseline condition of each colony at its time of collection. Each of the remaining three explants was randomly assigned to one of three environments: either returned to its original environment or transplanted to one of the other two. Colonies were attached to porcelain tiles using Portland cement, after our two initial efforts to secure them

(with stainless steel tapcon screws and a Portland mortar mix) proved infeasible.



Source of variation	df	F_{crit}	Interpretation of significant effect
Population of origin (P)	2	3.35	Ecotypic variation (local adaptation).
Growth environment (E)	2	3.35	Environmental influences differ from site to site.
P x E	4	2.73	Population by environment interaction; likely if stressors greater at HWO3 or PE3 compared to FTL3.
Residual	27		
Total	35		

Figure 8. Design and skeleton analysis of variance table for the reciprocal transplant experiment.

The next day, we secured the plates to the ocean floor using Portland cement. We also marked two additional colonies at each site to serve as unmanipulated controls. This experiment is still underway (Fig. 9), and we will assess colony condition in the fall, 2011, using our monitoring methods, including sampling for CDS and chemical analysis. We expect that some explants, particularly colonies from the FTL3 control site, may do poorly at the other sites.

In April and June, 2011, we photographed all coral colonies and estimated their surface area using ImageJ Software (National Institutes of Health, Washington, DC). In this software, we outlined a polygon that encompassed the live coral tissue and determined the number of pixels contained within it. We repeated this procedure with the tiles (Fig. 9), and used them to define the number of pixels per cm^2 in each image. We then estimated each coral's surface area as:

$$\frac{\text{Number of pixels comprising coral colony}}{\text{Number of pixels comprising tile/tile area in } cm^2}$$

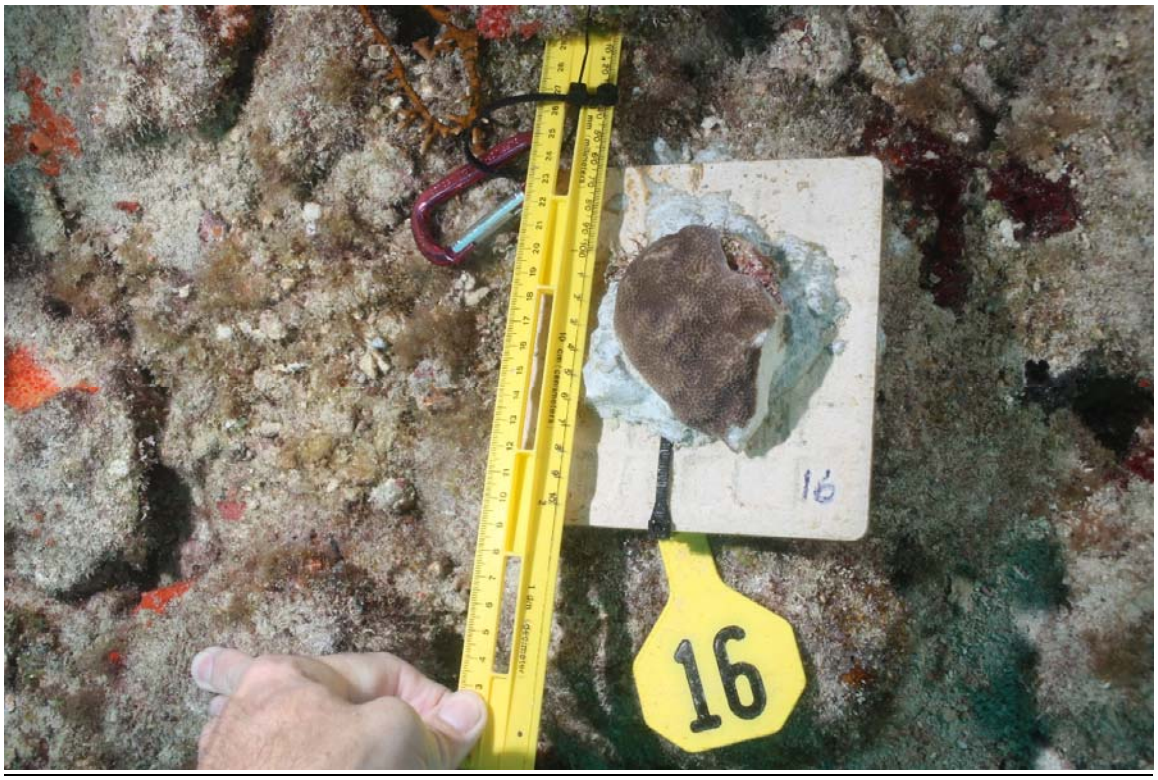


Figure 9. Explant of the coral P. astreoides from PE3 transplanted to HWO3.

We will continue to monitor these experimental *P. astreoides* for at least 1 y. We also will obtain samples from four colonies originating from a given site, and that survived at their natal station and both stations to which they were transplanted. This will yield a total of 36 coral explants and permit powerful tests of the null hypotheses of no ecotypic variation, no variation in environmental influences, and no population of origin x environment interactions (Fig. 8). If conditions indeed are stressful at HWO3 and PE3, we expect a reduction in stress biomarkers when explants are grown at FTL3; we also expect that stress biomarkers will be upregulated when colonies from FTL3 are transplanted to HWO3 and PE3, but not when they are returned to their natal site.

2.10 Statistical analyses

We used repeated measures analysis of variance (ANOVA) to test the null hypothesis that coral richness, evenness, diversity, and percent live cover did not vary between depths, among stations, over time, or with their interactions. Strong currents and a flooded camera prevented us from videotaping Station C3 in 2008 so this year was not included in the analysis. We included data from 2005 (see Fauth et al. 2006) to increase the time span of our analysis to include 2005, 2007 and 2009.

We used one-way ANOVA to test the null hypothesis that hatchability and viability of brine shrimp, and sea urchin fertilization success were independent of water source, either among the eight stations or the artificial seawater control. We also used one-way ANOVA to test the null hypothesis that larval settlement in the 2009 experiment was independent of substrate and rock origins. Because lesion regeneration rates were very heteroscedastic, we used Welch ANOVA to test the null hypothesis that lesion regeneration rates were independent of stations.

We used one-way ANOVA to test the null hypothesis that mean biomarker levels differed significantly among stations. We $\log_{10}$-transformed all biomarker data except ubiquitin to attain residuals that were randomly and normally distributed. We

We used two-way factorial analysis of variance to test the null hypothesis that percentage change in size *P. astreoides* in the reciprocal transplant experiment was independent of station of origin, station deployed, and their interactions (Fig. 8). We compared colony size between April and June for this analysis, and caution that corals may not have grown enough during this short period to yield substantive results. This time spanned the reproductive period of *P. astreoides*, which is a stressful time for corals. It also did not encompass the wet season or elevated water temperatures, which can have strong direct and indirect (e.g., through algal overgrowth) effects on coral condition.

We used analysis of covariance (ANCOVA) to test the null hypotheses that larval settlement in the 2011 experiment was independent of the station in which settlement plates were conditioned. Covariates in this analysis were positions of aquaria within the laboratory (scored 1-12) and the day on which settlement was quantified (1-3). Position within the same NCRI laboratory influenced coral physiological responses in our previous study (Fauth et al. 2006).

We used multivariate analysis of variance (MANOVA) to test the null hypothesis that percent cover on sediment traps did not vary among stations. Because some sediment traps were overturned by storms or anchors, or moved by recreational divers, we combined data from 2009 and 2010 to obtain at least two replicates per station. We used repeated measures ANOVA to test the null hypothesis that DNA AP sites did not vary between years, among stations, or with their interactions. The same individual coral colonies were samples at each station in both years, mandating use of a repeated measures design. We used ANOVA to understand significant sources of variation in both these multivariate analyses.

We evaluated the suitability of all analysis of variance tests by confirming that residuals were random, normal and independent, by examining plots of

residuals versus the predicted values. When necessary, variates were transformed to meet analysis assumptions. All statistical analyses were performed using JMP V. 4.0.4 or higher (SAS Institute, Inc., Cary, NC, USA), with $\alpha = 0.05$ for all hypothesis tests.

3 RESULTS AND DISCUSSION

Overall, the results of this project agreed well with our previous study (Fauth et al., 2006, Dustan et al., 2008), the co-located human fecal contamination study of Futch et al. (2011), and gene expression profiling off the Miami-Dade coast (Edge, 2008), which used a similar sampling design. We observed obvious differences between mid-water and deeper sites, among stations, and over time. Differences extended from the level of the coral reef community to those of individual coral colonies, their cellular physiology, and DNA condition. Laboratory experiments had variable results, including poor performance of some reference stations, making results of the reciprocal transplant experiment critical for determining causality. Below, we summarize the results of our current project and discuss their implications. We begin at the level of the coral reef community and proceed to lower levels of organization, and then to the abiotic factors and experimental results.

3.1 Ecosystem Assessment

Stations near ocean outfalls consistently had more bare, uncolonized substrate than those distant from the two outfalls (repeated measures ANOVA $F_{1,4} = 13.98$, $P < 0.021$). The difference ranged from 10% more bare substrate in 2005 to twice as much in 2007 (Fig. 10). No other substrate category differed significantly among depths or with proximity to inlets or outfalls. Percent coverage by scleratinian corals was very low in these three years, ranging from 0.31% at PE2 to 2.58% at HI3. This range of percent coverage is typical for the region and not different from average at other Broward 2nd and 3rd reef sites over the last 9 years (Louis Fisher, *personal communication*). Between 2005 and 2009, percent coral cover declined at six sites (95% CI: -0.08 to -0.72), with cover statistically remaining constant only at PE2 and PE3 (95% CI: -5.52 to 6.69).

Coral richness estimated by point counting was significantly higher at midwater than at deepwater stations (repeated measures ANOVA $F_{1,4} = 8.96$, $P < 0.041$). On average, the number of coral species point-counted on midwater stations was 50% higher than the number at deepwater stations (5.4 ± 0.63 versus 3.6 ± 0.56 species). Coral evenness, diversity, and apparent species richness (Jost 2006) did not differ significantly among years or stations. Adult corals present in both 2004 and 2007 and as juveniles in 2007 were the principal species present on the reef: *Montastraea cavernosa, Stephanocoenia intersepta, Solenastrea bournoni, Siderastrea*

siderea, Dichocoenia stokesi, Porites astreoides, and *Meandrina meandrites*. Species present as adults but not juveniles may be undergoing local reproductive failure, not contributing to future generations, and therefore essentially remnants populations. Species found only as juveniles and not adults may originate upstream and be unable to survive for more than a few years under present environmental conditions.

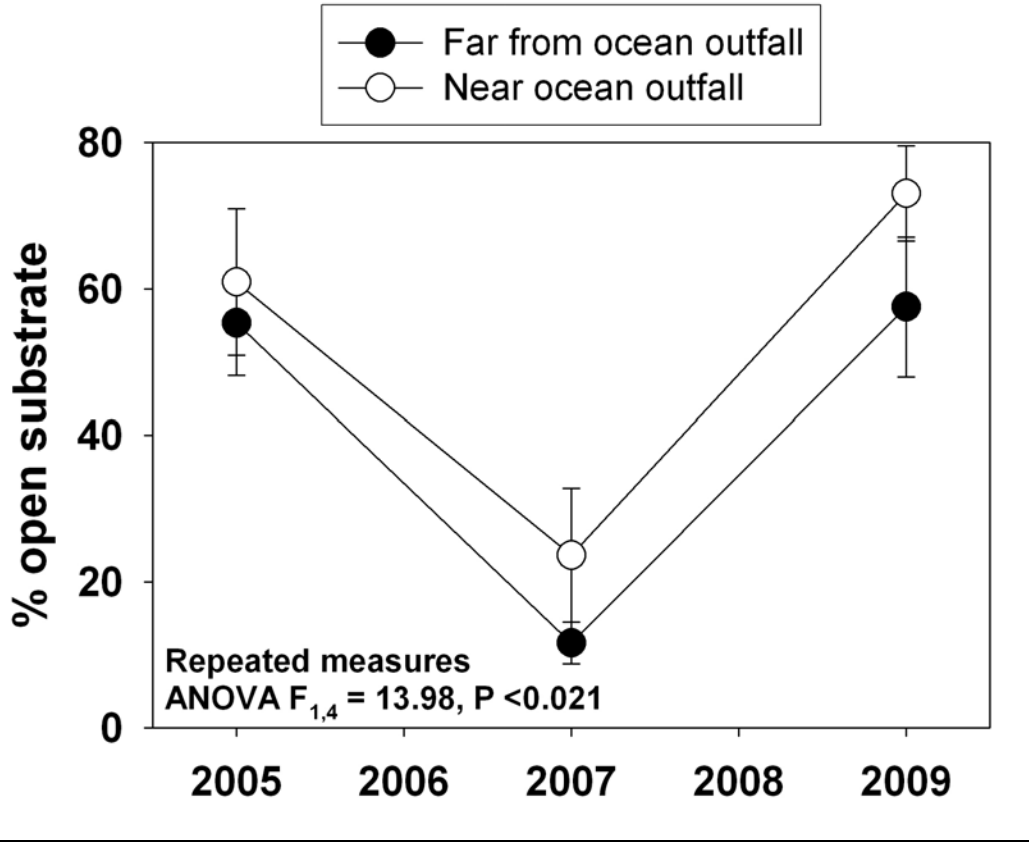


Figure 10. Percentage of open substrate as a function of proximity to ocean outfalls. Data from two, 15-25 m video transects recorded in 2005, 2007 and 2009.

3.2 Coral Regeneration Rates

Our best data on regeneration rates of *Porites astreoides* is from the initial sampling conducted March 5-7, 2008. At that time, we removed small (95% CI: 1.32 – 1.45 cm^2), circular biopsies of tissue and underlying coral skeleton and filled the holes with non-toxic modeling clay. On June 11–12, 2008, we re-measured these sampling lesions and detected no significant differences among stations in mean regeneration rate (Welch ANOVA $F_{7,\ 13.7} = 0.41$, $P > 0.87$). However, regeneration rates were highly variable within stations, largely due to a few colonies whose lesions expanded instead of regenerating (Fig. 11).

Combined with data from our previous study (Fauth et al., 2006, Dustan et al., 2008), a pattern emerged: colonies at all deepwater stations except PE3 were less likely to completely heal after lesion formation than colonies at the midwater stations (Table 2). We also found this pattern with another coral (*Montastraea annularis* sp. complex) within Biscayne National Park and the Florida Keys National Marine Sanctuary (Downs et al. 2002, 2005; Fisher et al. 2007). Colonies in deeper sites were less likely to heal, lost more live tissue, and declined in size compared to those in shallower sites.

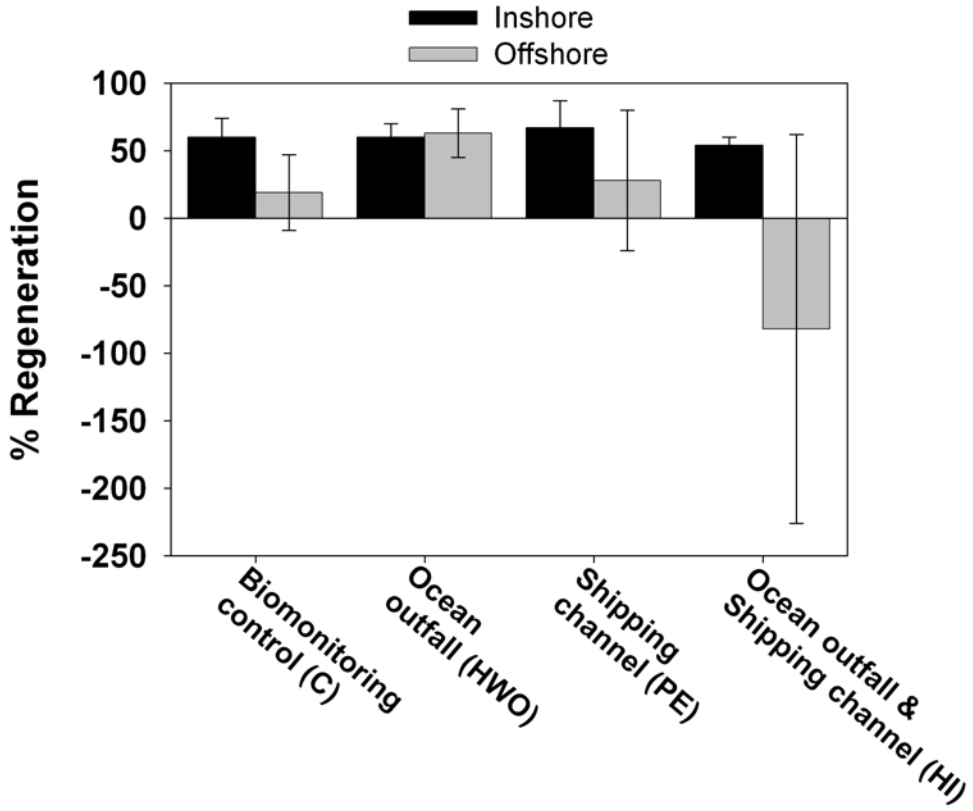


Figure 11. Mean (± 1 SE) percent lesion regeneration between March and June, 2008, as a function of depth, station and potential sources of land-based pollution off Broward County, Florida.

We did not observe disease in any of the corals we sampled or marked, and only one colony partially bleached (Colony 73 at C2 in June, 2008). A few colonies were overgrown with algae, especially in March 2007 at HI2 and PE2 (4 of 6 and 4 of 5 colonies sampled for cellular diagnostics, respectively), when *Lyngbya* covered substantial portions of our stations and there was less open bottom (Fig. 10). A few colonies also had bite marks from parrotfishes and other grazers.

Table 2. Summary of lesion regeneration rates of Porites astreoides after sampling for cellular diagnostic analyses. For regeneration rates, + depicts a 95% CI with positive values and a 0 indicates it overlapped zero.

Station	Regeneration rate in 2005-2006	Regeneration rate in 2008	Status in May 2009
HI2	+	+	3 of 5 healed
HI3	0	0	10 of 12 healed
C2	+	+	8 of 9 healed
C3	+	0	3 of 3 healed
PE2	0	+	5 of 5 healed
PE3	0	+	5 of 8 healed
HWO2	+	+	10 of 10 healed
HWO3	0	0	4 of 5 healed, 6 colonies missing

3.3 Cellular Diagnostics

We found statistically significant variation in DNA AP Site among stations, between years (2007 and 2009) and their interactions (Repeated measures ANOVA $F_{7,14} = 6.27$, $P < 0.002$). In 2007, corals at HWO2 had significantly fewer DNA AP sites than those from the other midshore stations (Fig. 12). Colonies of *P. astreoides* from HI2 had significantly more DNA damage than those at HWO2 and all deepwater stations except HWO3. The remainder of the stations had intermediate levels of DNA damage. In 2009, the pattern was very different: corals at PE2, PE3 and C3 had an order of magnitude more DNA damage than those at C2, with the remaining stations indistinguishable from the others (Fig. 12).

Overall, mean levels of DNA AP sites were slightly higher in 2007 than in 2009 and were consistently higher at HI2 and PE2, the two midwater sites near shipping channels (Fig. 12). Other stations were variable, and only HWO2 had consistently infrequent DNA AP sites. Genetic damage to colonies of *P. astreoides* was more extensive in nearshore stations with nearby shipping channels, suggesting that run-off of pollutants from their extensively developed shorelines harmed colonies on nearshore reefs. However, overall frequencies of DNA AP

sites were 1-2 orders of magnitude lower than we observed at six stations off St. John, U.S. Virgin Islands (Downs et al. 2011). Mass mortality of corals (*P. porites*, *M. annularis* and *A. palmata*) occurred at these USVI stations, which were within embayments that had more limited water circulation than our Broward County stations.

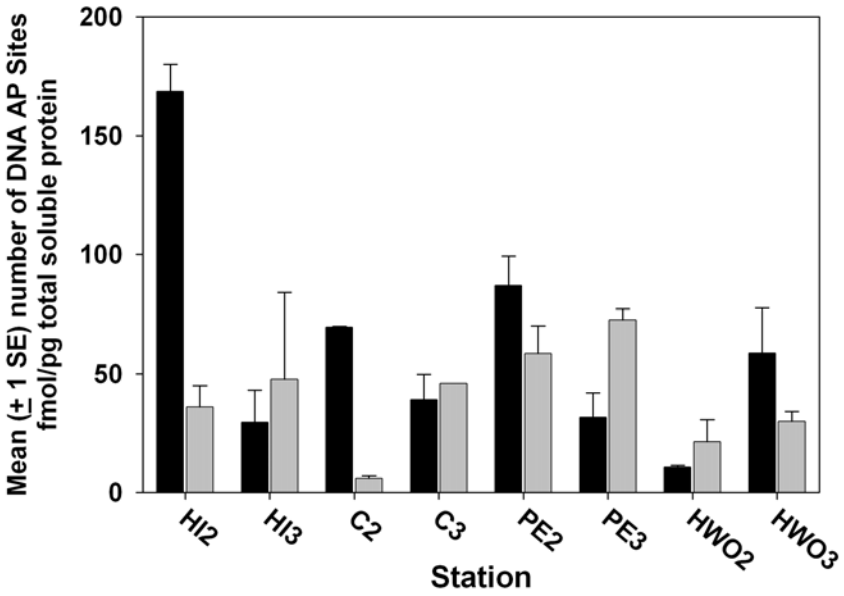


Figure 12. Mean ± 1 SE number of DNA AP sites in colonies of Porites astreoides sampled off Broward County, FL in 2007 (black bars) and 2009 (gray bars).

Repeated measures ANOVA also showed significant variation among stations, and with the station x time interactions, on mean concentrations of catalase and Cu/ZN SOD (all $F > 4.46$, $P < 0.02$). Both antioxidant enzymes had similar patterns: high levels at PE2 and PE3 in both years, and additional spikes at HI2, FTL2 and HWO3 in 2007 and at HI3 in 2009. However, ubiquitin concentrations did not vary significantly among stations, years, or their interactions. Combined, these results suggest that *P. astreoides* experienced stress from reactive oxygen species that caused upregulation of antioxidant enzymes. This defensive reaction, which dismutated oxygen radicals into hydrogen peroxide and then to water and molecular oxygen, protected coral proteins from degradation but were not as effective protecting DNA.

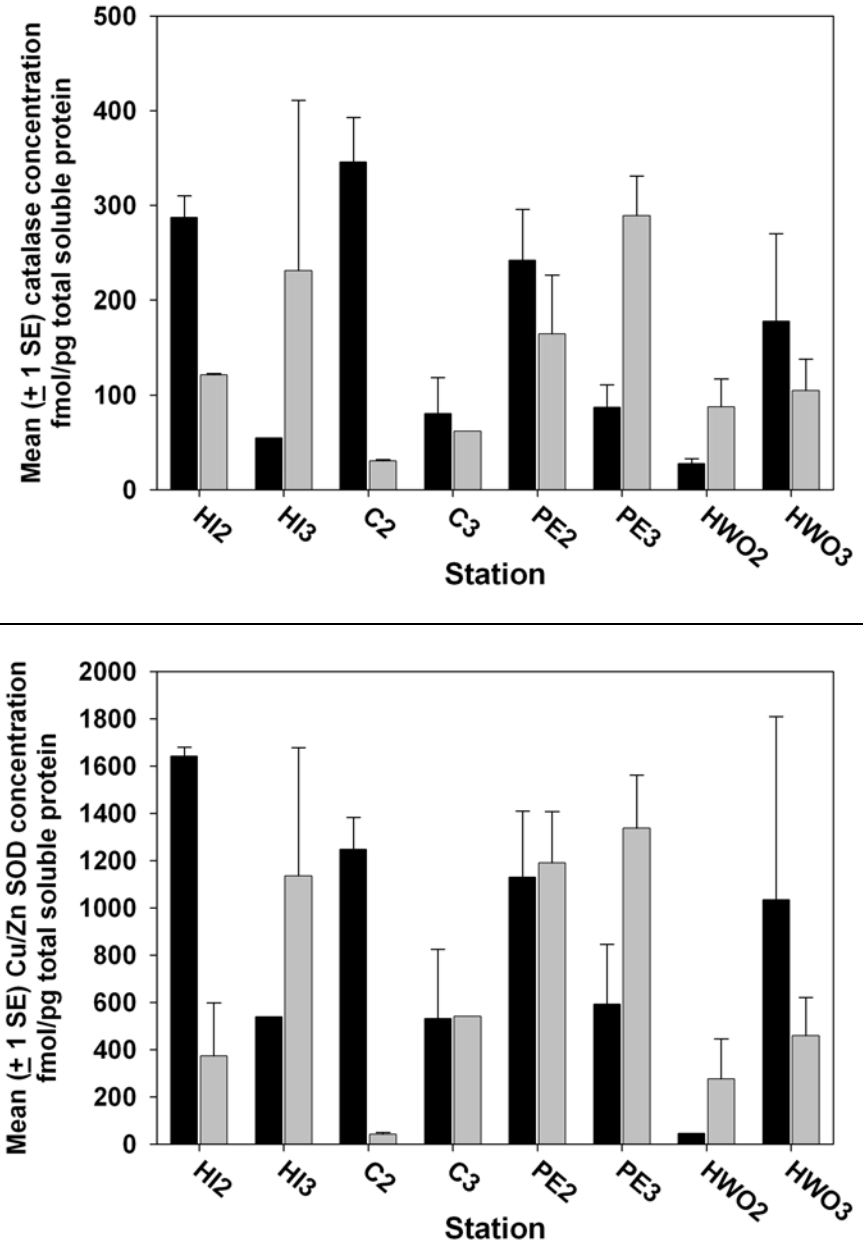


Figure 13. Mean ± 1 SE concentrations of catalase (top) and Cu/Zn SOD in colonies of Porites astreoides sampled off Broward County, FL in 2007 (black bars) and 2009 (gray bars).

3.4 Temperature and Sedimentation

Temperature data were limited by loss, removal and failure of data loggers and by deployment times that exceeded their limited memory. The data set therefore contained some major gaps. Nevertheless, the available data show similar temperature profiles at all stations below the ~30° C threshold that causes coral bleaching. The most prominent event was a cold snap in early January, 2008 (Fig. 14), which affected all midwater stations except PE2 more than the deepwater stations. Because temperatures were highly correlated among stations, thermal differences are unlikely to be responsible for differences among them in coral condition.

Similarly, sedimentation data also were limited by removal and overturning of sediment traps and by deployment times that were too long, which allowed bivalves and other invertebrates to clog the openings. However, traps deployed for long periods were excellent, standardized substrates for evaluating the balance between growth and herbivory, which forms the conceptual basis of the AGRRA and other reef assessment protocols. Multivariate analysis of variance on the vector {bare PVC + scrapes, barnacles, all other invertebrates, red algae, green algae, filamentous algae, calcareous algae} revealed significant variation among stations (Wilks' $\lambda = 0.0478$, $F_{49,\ 116.1} = 1.94$, $P < 0.002$). Univariate tests showed this was mainly caused by significant variation among stations in the percent coverage by red algae (ANOVA $F_{7,28} = 3.55$, $P < 0.008$). Tukey's Honestly Significant Difference showed that sediment traps at HWO3 were 65% covered by red algae, significantly more than at any other station. Stations PE2 (31%) and FTL3 (15%) were statistically indistinguishable from HWO3 in percent coverage with red algae. Smothering by algae is an important factor limiting growth of corals, particularly when algal grazers are limited. Regeneration of coral lesions was very limited at HWO3 during this and our previous study, and algae already are colonizing tiles there in our reciprocal transplant experiment.

3.5 Contaminant Analyses

Frozen samples of sediment, reef and surface water, and coral tissue are available for contaminant analysis. Because funding was not available for contaminant analysis, we have to choose samples wisely for these expensive assays. Based on results of lesion regeneration rates, percent cover on sediment traps, the toxicity experiments, and initial results of the reciprocal transplant experiments, the most useful comparisons of contaminant loads likely will be between HWO3 and C3. These stations were the most divergent in almost all of our investigations.

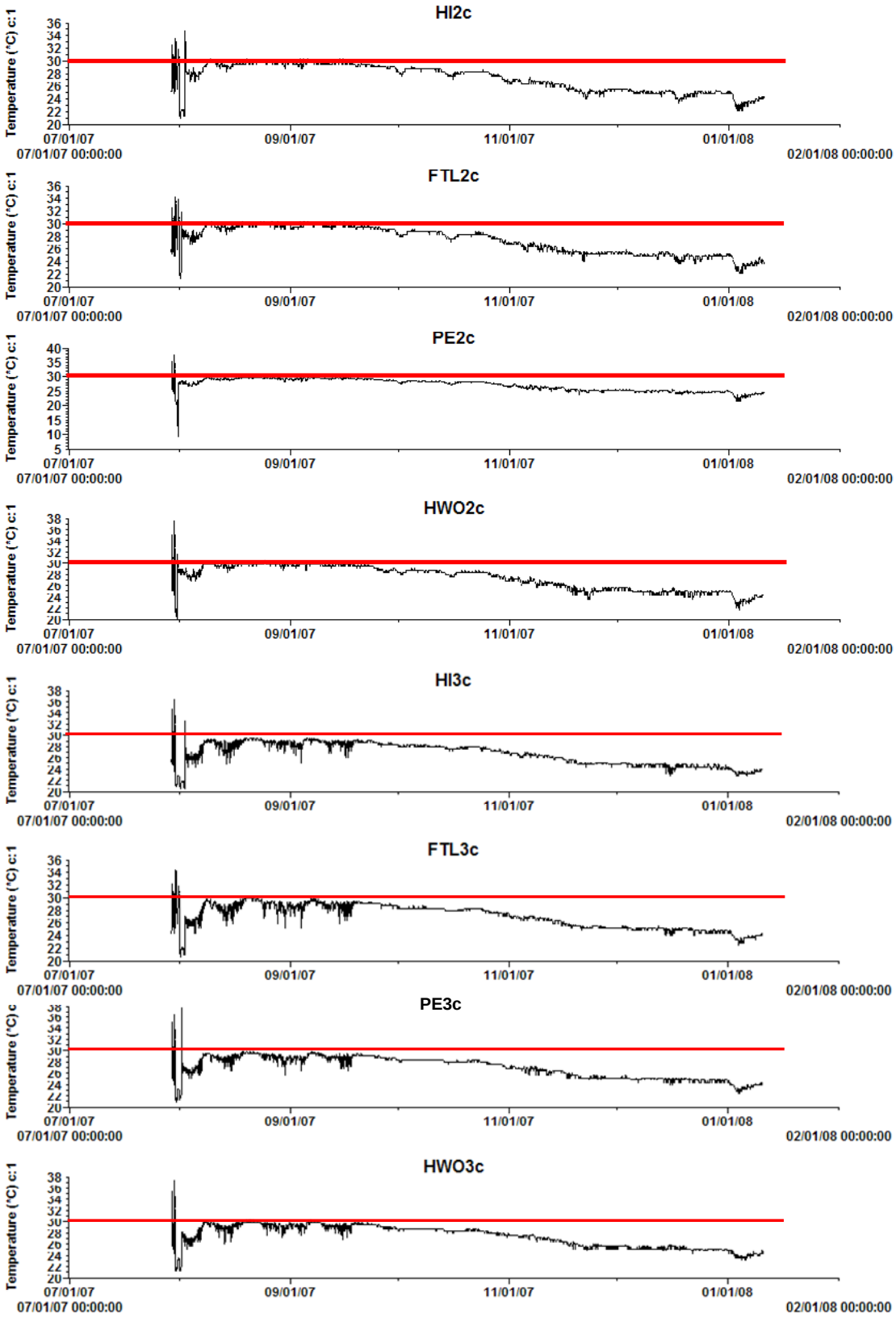


Figure 14. Temperature profiles at all stations from September 1, 2007 – February 1, 2008.

3.6 Experiments on Larval Coral Settlement

Settlement of corals was very low in the 2009 experiment. One-way ANOVA indicated that there was no significant difference in larval settlement among treatments ($F_{2,6}$ = 0.20, P > 0.82). Larval *P. astreoides* settled in only one of the three replicate aquaria in two treatments, the control (9 larvae) and C3 (5 larvae). A total of 4 larvae settled in the HI3 treatment, at least one in each aquarium.

Settlement rates in this study were very low compared to other experiments performed in Dr. Moulding's lab (including the 2011 experiment; see below). One reason settlement rates were so low was sedimentation on the settlement plates. We did not quantify sediment coarseness, but treatments obviously differed in sediment quality; the aragonite sand used in the control treatment was the coarsest, and sand from site C3 was the finest. Although great care was taken to keep plates clear of sediment, any water movement (e.g., water changes necessary to maintain water quality while the pumps were off) stirred up sediment. When plates were cleared, the sediment resuspended and eventually settled back onto the plates. Because coral larvae prefer hard surfaces, sediment deposited on the plates may have inhibited coral settlement. Sediment has detrimental effects on coral settling and survival, including on reefs off the southeast Florida coast (Vargas-Angel et al. 2007, and references therein; Jordan et al. 2010). At our HWO3 station in May, 2009, we could not locate marked coral colonies on the east side of this transect despite finding their tags. On our next dive, the sediment was gone and we located the colonies. This type of intermittent burial is consistent with the hypoxia/reperfusion (= smothering and resuscitation) response reported in our initial study (Fauth et al. 2006). On the same dives, colonies on the west side of the HWO3 transect were not buried, illustrating the potentially very local scale (< 25 m) of sedimentation impacts. We also observed considerable flocky sediments at PE3 in March, 2008.

Another potential reason settlement rates were so low in our 2009 experiment may be the short conditioning time of the terracotta settlement plates. Conditioning is placement of settlement substrata in seawater before larval or gamete release, and allows development of biofilms, which are believed to provide larval settlement cues (Webster et al., 2004). Conditioning times used in experiments vary, but generally the minimum is a couple weeks. In our study, we only conditioned settlement plates for 3 d because the larval release date of *P. astreoides* was approaching and earlier dive trips were postponed due to poor weather. Thus, longer conditioning times were not possible.

Larval settlement rates were much higher in the 2011 experiment, averaging 56.7% across all treatments. Analysis of covariance revealed significant variation due to the day in which settled larvae were counted (Table 3), with plates counted later having more larvae settled. This is a common occurrence because

some larvae settle later than others; because of their small size and cryptic appearance (see cover photo), it was impossible to count larvae on all 36 plates in one day.

Table 3. Results of analysis of covariance on percentage settlement of larval Porites astreoides as a function of position within the laboratory, day settlement was scored, the source of plate conditioning, and its interaction with day scored. Two models are shown, one that includes the nursery plates and the other without them. Table entries are sources of variation, degrees of freedom (df), sums of squares, F ratio (F) and p-value (P).

With nursery plates included; model $R^2 = 0.964$

Source	df	SS	F	P
Position	1	157.34	8.71	0.060
Day	1	306.58	16.97	**0.026**
Station	3	480.56	8.87	0.053
Day x Station	3	270.17	4.99	0.110
Error	3	54.19		
Total	11	1486.42		

Without nursery plates; model $R^2 = 0.952$

Source	df	SS	F	P
Position	1	138.11	9.96	**0.034**
Day	1	428.62	30.91	**0.005**
Station	2	273.02	9.84	**0.028**
Error	4	55.46		
Total	8	1153.36		

Analysis of covariance also revealed substantial, but not statistically significant, variation due to the station in which plates were conditioned and the position of aquaria within the laboratory (Table 3, top). After adjusting for the day on which larvae were counted and position in the laboratory, a greater percentage of larvae tended to settle on plates conditioned at stations HWO3 (65 ± 2%) and C3 (60 ± 3%) than at PE3 (52 ± 3%) or in the NCRI nursery (49 ± 2%). Combined, all factors in the ANCOVA model accounted for 96.3% of the total variation in larval settlement. Removing nursery plates from the model eliminated the need for the day x source interaction term and all three remaining terms explained significant variation (Table 3, bottom). As in the previous analysis, adjusted settlement was higher on plates conditioned at stations HWO3 (66 ± 2%) and C3 (61 ± 2%) than at PE3 (52 ± 2%). This result suggests that differences among sites in suitability for coral settlement are real. In our previous report (Fauth 2006), we remarked that inlets are challenging environments for *P. asteroides* because we did not find any colonies at our initial mid-depth dive site off Hillsborough Inlet. We had to move across the inlet to find *P. astreoides* at the station that became HI2. Similarly, at the request of the Florida Department of Environmental Protection, we searched for *P. astreoides* at the opposite end of our PE2 transect and did not find any colonies. Limited numbers of coral larvae and laboratory aquaria prevented us from testing settlement plates from these mid-water stations; we suggest repeating this experiment with them. Settlement of larval corals is reduced when appropriate cues are absent and in the presence of inhibitors (e.g., Kuffner et al. 2006, and references therein).

3.7 Toxicity Identification Experiments

We found no significant differences in brine shrimp hatchability or lethality in treatments with pore water and reef water collected in August, 2007 or March, 2008. Brine shrimp are notably hardy organisms; the standard technique for decapsulating their eggs includes soaking them in freshwater for 1 h, adding an equal volume of bleach, rinsing eggs that settle on the container's bottom with fresh water, and then hatching them in seawater having the desired salinity (Watson and Yanong 2002). Lack of toxicity in these experiments suggests that a highly acute stressor was not present in pore water or reef water at any of our eight stations.

We found significant differences in sea urchin fertilization success between artificial seawater (ASW) controls and pore, reef and surface waters (Table 4, Fig. 15). In some tests, we also found significant differences among stations, but often the lowest fertilization success was in waters from the biomonitoring control sites, C2 and C3 (Table 5). These results are consistent with the observation of high stress biomarkers at all eight stations we studied compared to corals in the Bahamas (Fauth et al. 2006, Dustan et al. 2008). Futch et al. (2011) also found evidence of human fecal contamination at these eight stations: "the overall

prevalence of enteric viruses in this study was remarkably similar across all sites and potential sources, suggesting that contamination may be widespread, or that contaminants may be quickly transported and mixed, along the coast of this densely populated area." Lack of consistency among our laboratory tests reinforces the theory that reefs off the southeast Florida coast experience varying, intermittent, chronic stressors, which makes identifying causal relationships difficult (Fauth et al. 2006, Dustan et al, 2008).

Table 4. Summary of results of sea urchin fertilization assays conducted on pore, reef and surface waters collected at eight stations off Broward County, Florida, and artificial sea water controls. Station abbreviations are as in the text. NS = no statistically significant differences among treatments at $\alpha = 0.05$.

Date	Water source	Sea urchin species	Statistical results	P
June 2008	Reef and pore water along HWO transect*	*Lytechinus*	NS	>0.29
June 2008	Reef water	*Lytechinus*	NS	>0.45
May 2009	Reef water	*Arbacia*	% developing to pluteus stage in HWO2 water overlapped that of no-sperm control	**< 0.002**
May 2009	Pore water	*Arbacia*	% developing to pluteus stage in HWO3 and C3 water differed significantly from the ASW control	**< 0.003**

*Surface water samples were collected from the outfall boil, at HWO2 and HWO3, and midway between them.

In August, 2007, we discovered significant differences among stations in pore water salinity (Fig. 16), which were normal at all sites (95% CI: 34.8 – 36.4 ppt) except HWO2 (26 ppt), HWO3 (3-12 ppt) and one sample from PE3 (17 ppt). These low salinities are lethal to sea urchin embryos, which are very sensitive to osmotic pressure (Figs. 16, 17). Salinity of reef-level water samples collected at the same time did not vary significantly among sites (one-way ANOVA $F_{6,12} = 1.92$, $P > 0.41$), and averaged 36.9 ppt. Concurrently, human enteric viruses were isolated from the Port Everglades channel and at PE3 (Futch and Griffin, 2008). These signals of fecal contamination are further evidence of intermittent, localized, water-borne stressors affecting the southeast Florida reef community.

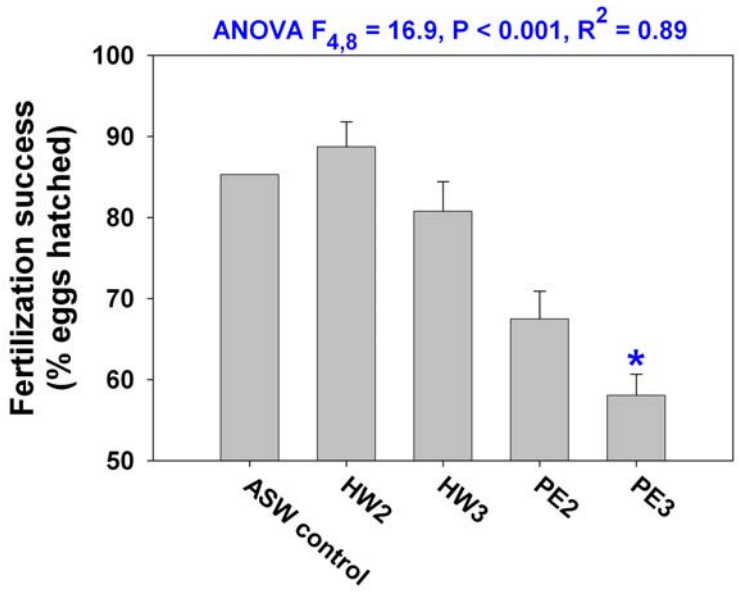


Figure 15. Percentage success (mean ± 1 SE) of sea urchin gametes fertilized in reef water from four stations versus the artificial sea water control.

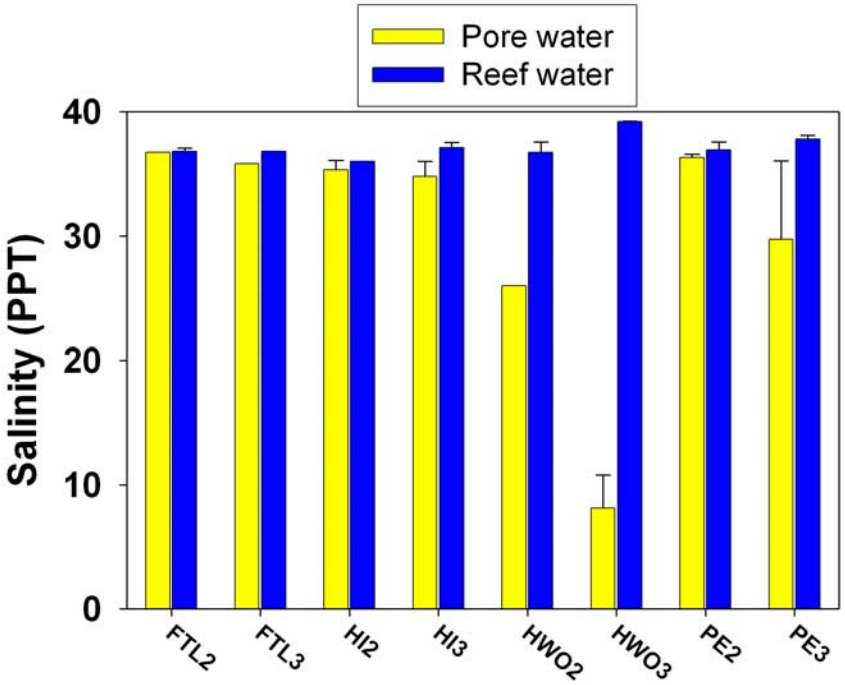


Figure 16. Salinity (mean ± 1 SE, in ppt) of water sampled at two depths at eight stations off Broward County, Florida. Abbreviations as in the text.

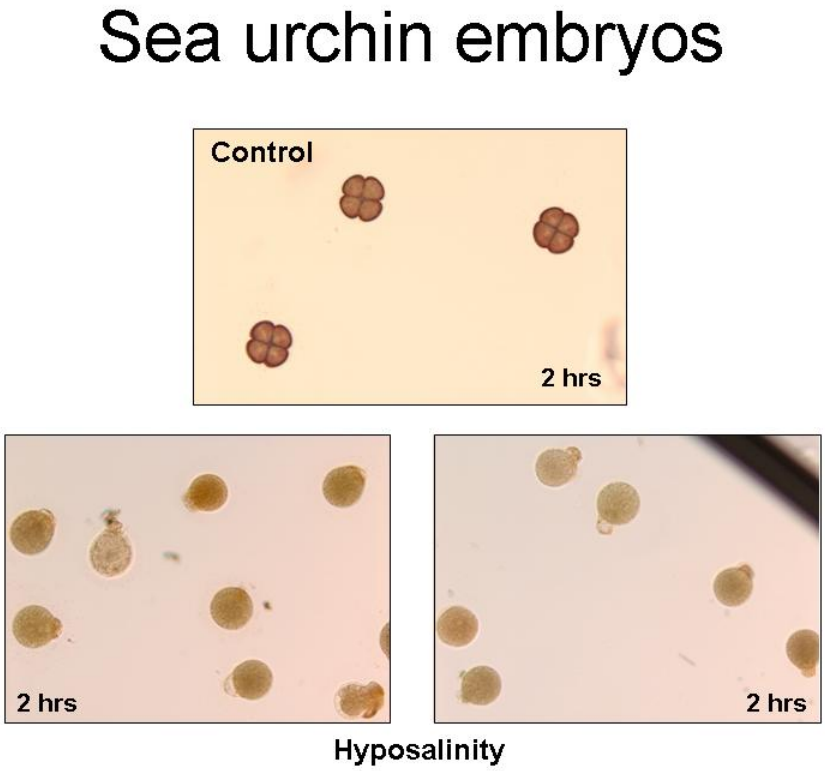


Figure 17. Development of sea urchin embryos under conditions of (top) normal and (bottom) hyposalinity.

3.8 Reciprocal Transplant Experiment

After one full year of growth, ANOVA found no statistically significant variation attributable to source of coral colonies, the station to which they were transplanted, or their interactions (all $F > 1.32$, $P > 0.28$). Averaged among all stations, surface area of colonies increased 14.8% (95% confidence interval: 1.1-28.5%). This experiment remains underway and we plan to continue monitoring it as long as possible.

4 CONCLUSIONS AND RECOMMENDATIONS

Southeast Florida's coral reef ecosystem is a rich natural and economic resource that is vulnerable to atmospheric, marine and land-based pollution from sources that range from local point sources (ocean outfalls) to regional (elevated sea-surface temperature) and even global forces (ocean acidification). The goal of our project was to identify and trace stressors affecting coral condition and to use experiments to test causal relationships. At the ecosystem level, we found very low percentages of live scleratinian coral cover in 2005, 2007 and 2009. Between 2005 and 2009, percent coral cover declined at six stations and remained about

constant at two others (PE2 and PE3). Mean percentage of live coral cover did not increase at any station, which is cause for concern. In addition, colonies of *P. astreoides* are not growing early in the reciprocal transplant experiment. Low and declining coral cover is symptomatic of chronically stressed coral reefs, which in just decades can be reduced to rubble or bare substrate. We found that stations near ocean outfalls consistently had more bare, uncolonized substrate than those distant from the outfalls. The difference ranged from 10% more bare substrate in 2005 to twice as much in 2007. In the laboratory, few sea urchin gametes developed to the pluteus stage when grown in reef water collected from HWO2 and pore water collected from HWO3. And in 2007, we detected pore water with very low salinity at HWO2 and especially HWO3. These results indicate that waterborne contaminants and freshwater intrusion negatively affected corals there. Futch et al. (2011) verified the presence of human enteric viruses, which are markers of human fecal contamination, at our stations during this period.

We caution that our own assessments are conservative because we could not safely dive under heavy east winds that can drive water from the ocean outfall shoreward, over our stations. Smothering by sedimentation is a potential selective agent at HWO3, where on one sampling date we could not locate colonies of *P. astreoides* at one end of this transect because they were buried under sediment. This type of intermittent burial can induce physiological hypoxia/reperfusion (= smothering and resuscitation) responses, which we noted in our previous study (Fauth et al., 2006) and which are metabolically costly to maintain. Sedimentation also prevented larval *P. astreoides* from settling in our first laboratory experiment.

Even more obvious than increased bare substrate was the lack of live *P. astreoides* at the opposite end of the 25 m PE2 transect. In our previous study (Fauth et al. 2006), we also did not find any live *P. astreoides* at the site initially selected for HI2 and had to use detailed LIDAR maps to find a suitable replacement. *Porites astreoides* is a notoriously tough, weedy species, and its absence at one-half of our initial midwater stations along two very different shipping channels indicates conditions were unsuitable for it to establish and grow. *Porites astreoides* at both these stations consistently had more DNA AP sites than at the other stations, which shows attacks from reactive oxygen species damaged their genetic material despite elevated levels of protective antioxidant enzymes. DNA damage could lead to reproductive failure at these sites, which drain extensive urban and industrial areas. However, *P. astreoides* at these sites regenerated over biopsy sites well, and may be locally adapted to stressors at these sites.

In the laboratory, *P. astreoides* larvae had reduced settlement on substrates conditioned at PE3, and sea urchin gametes had reduced fertilization success in reef water collected there. These results suggest that waterborne contaminants

and an unsuitable benthic community limit establishment of *P. astreoides* off the Port Everglades channel. Futch et al. (2011) mapped the concentrations of fecal indicators emanating from this channel in July 2007.

Concentrations of two diagnostic biomarkers (catalase and Cu/Zn SOD) varied significantly among sites but were inconsistent among years. Both are antioxidant enzymes that protect cells from damaging reactive oxygen species (e.g., oxygen radicals, hydrogen peroxide). High levels at PE2 and PE3 in both years suggest contaminants were transported to the reef via the channel (c.f., Futch et al., 2011). Additional spikes at HI2, HI3, and HWO3 link damage to other point sources of pollution. In contrast, the spike at FTL2 in 2007 may be due to an unidentified point source, a non-point source, or transport and mixing from elsewhere. Ubiquitin concentrations did not vary significantly among stations, years, or their interactions. Combined, these results suggest that *P. astreoides* experienced stress from reactive oxygen species that mobilized antioxidant defenses. This physiological defense protected coral proteins from degradation but were not as effective protecting DNA. However, concentrations of catalase and Cu/Zn SOD were lower off Broward County than in *P. astreoides* sampled within the same time span off St. John, U. S. Virgin Islands. There, several species of corals experienced mass mortality and *P. astreoides* was challenged at six sites by different stressors, ranging from fecal coliforms to polyaromatic hydrocarbons and semi-volatile organochlorines and nitrogen-based biocides (Downs et al., 2011). The remaining biomarker we assayed, ubiquitin, is indicative of protein turnover and was orders of magnitude higher in *P. astreoides* off Broward County compared to St. John. Ubiquitin is expressed at high levels during colony growth but unfortunately lesion regeneration was not measured in the Downs et al. (2011) study.

At the level of individual coral colonies, we found no significant differences among stations in mean regeneration rates, which were highly variable within stations. However, evaluating these results alongside those of our previous study (Fauth et al., 2006, Dustan et al., 2008) reveals colonies at all deepwater stations except PE3 were less likely to completely heal after lesion formation than colonies at the midwater stations. *Porites astreoides* at all deepwater stations except HWO3 had regeneration rates that did not differ significantly from zero, and most colonies in the reciprocal transplant experiment were losing live tissue. One common phase transition worldwide is replacement of distressed coral reefs by algal communities (e.g., Hughes, 1994). We found significantly more red algae on sediment traps at HWO3 than at any other station. Algal overgrowth often is associated with eutrophication, and we already noticed algae obscuring coral colonies in the reciprocal transplant experiment at HWO3 and PE3. In addition, deep water stations had significantly lower coral species richness than midshore stations from 2005-2009.

Overall, the results of this project are consistent with our previous study (Fauth et al., 2006) and those of independent researchers. We found evidence of negative effects of ocean outfalls (cf. Edge, 2008) and a shipping channel (cf. Futch et al., 2011) on coral reefs at multiple biological levels, ranging from genes to gametes to populations to the whole ecosystem. Some effects, such as burial by sediments, occurred on very small scales both spatially and temporally. Most were indicative of chronic rather than acute stress, and presage continued slow, sporadic declines of the southeast Florida coral reef. No station was exempt from at least one negative result. However, this outcome can be reversed by effective management, including closing the ocean outfalls as scheduled, reducing run-off onto the reef, and limiting discharge of agricultural, industrial and urban wastes from canals. To accomplish this, we recommend adopting a cohesive monitoring, research and management protocol similar to that employed by the Amphibian Research and Monitoring Initiative, ARMI (Fig. 18). Like coral reefs, amphibian populations are experiencing a global decline and the ARMI protocol is designed to "determine the scope and severity of the problem **and to investigate causes**" (http://armi.usgs.gov/index.php; web accessed 18 July 2011). It includes monitoring on a national scale at its base; coordinating scientific and management activities in its core; and detailed, long-term research conducted at a few sites at its apex. We strongly support co-locating scientific studies, measuring environmental conditions at the same place and time as biological responses, doing mechanistic studies that include multiple levels of the biological hierarchy (Fig. 2) and using an adaptive approach to managing southeast Florida's valuable coral reefs.

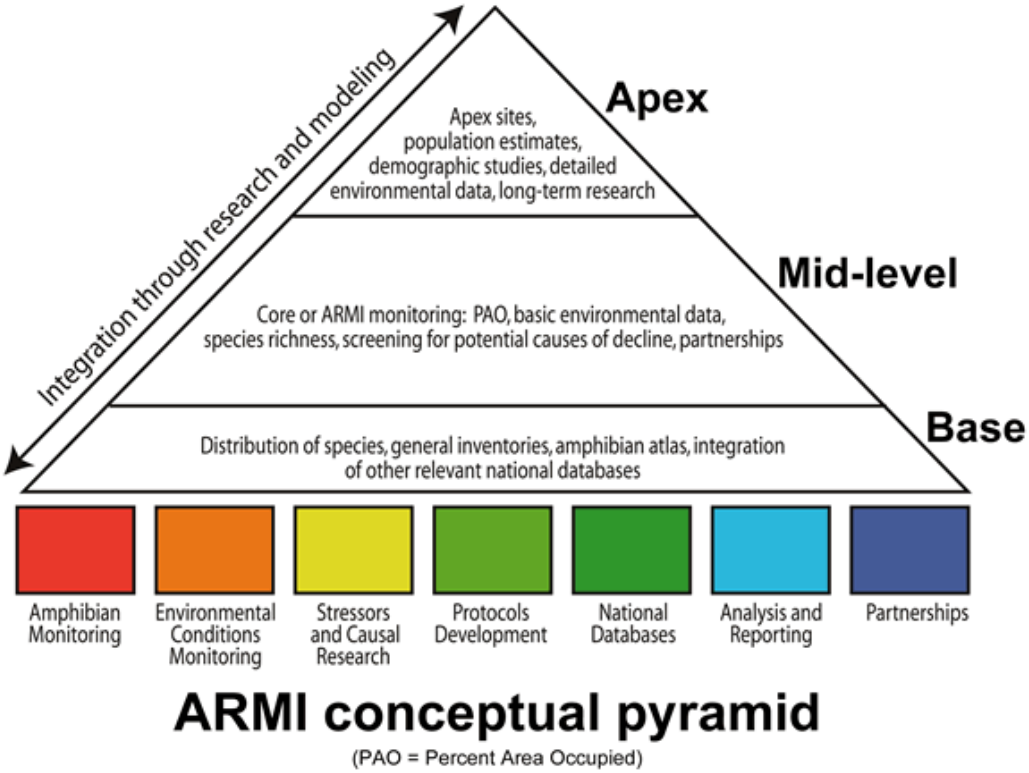


Figure 18. Conceptual model used in the Amphibian Research and Monitoring Initiative (ARMI), which should be adapted and used for coral reefs. The Southeast Florida Coral Reef Initiative (SEFCRI) already includes many of these elements but needs greater support.

The coral reefs off the southeast Florida coast are ecologically and economically valuable resources that merit increased protection and management. An adaptive management approach (c.f., Foxcroft 2004) is well suited to the busy southeast Florida coast. Multiple projects are often underway in a given region, providing an opportunity to gather information quickly and perform useful, management-oriented experiments. Reference sites for beach, harbor and management projects can overlap SECREMP, county, and biomarker stations, facilitating long-term comparisons and providing greater temporal resolution of biotic responses.

Providing scientifically valid recommendations to resource managers requires more than just monitoring. When humans are sick or injured, monitoring vital signs alone does not return them to good health. Monitoring assists diagnosis but cures happen because medical personnel take samples, compare them versus reference values, and perform interventions, which basically are experimental tests. If the first treatment fails, another experiment is attempted. A similar system of monitoring plus testing should be implemented for coral reefs throughout the SEFCRI region, with an emphasis on biotic parameters that

integrate stress-response relationships over time. Productive areas of investigation include:

- the role of biomagnification in transporting contaminants from ocean outfalls and other point sources to reef organisms
- using radiating transects to determine the reef area influenced by ocean outfalls, shipping channels, and other disturbances
- quantifying recovery of coral reefs near ocean outfalls (e.g., Delray Beach) that are closed compared to those that remain in use
- using transects and transplants across the tide line to determine how nutrient and contaminant loads in coastal rivers and canals affect reef organisms
- pilot projects that quantify the benefits to reefs of implementing agricultural best management practices in South Florida

Prioritizing stressor and causal research (the yellow box at the base of Fig. 16), coordination of data sharing and analysis (light blue box), and integrating research and modeling (the upper levels of the pyramid) will add to SEFCRIs strengths in monitoring, protocol development and partnerships, and provide a more effective means of understanding and managing southeast Florida's coral reefs.

5 ACKNOWLEDGMENTS

Bad weather, delays in special activity license reviews, and accommodating the life history of *Porites astreoides* lengthened this project well beyond its planned two-year duration. In fact, it's still not over because we will continue monitoring the reciprocal transplant experiment for at least another year! We thank the many contributors for maintaining their cheerful willingness to help, especially Louis Fisher, Joe Ligas, and Pat Quinn (Broward County Development and Environmental Regulation Division); Derek Chase, Sarah Green, Hayley Smith, and Greg Thompson (University of Central Florida); the Southeast Florida Coral Reef Initiative staff; members of the Land-based Sources of Pollution Technical Advisory Committee, especially Esther Peters who diligently read and commented on this report; and Richard Dodge and the many students and scientists who helped during our dives and provided support at Nova Southeastern University's Oceanographic Center.

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