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Using cellular diagnostics to link land-based sources of pollution with coral reef degradation in South Florida

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Abstract. Linkages between land-based sources of pollution and coral reef health were assessed at ecological, physiological and cellular levels at four paired inshore and offshore stations off Broward County, Florida, U.S.A.: a biomonitoring control site, a treated wastewater outfall, an inlet mouth, and a treated wastewater outfall adjacent to an inlet mouth. Live coral cover was <4% at all sites and most inshore sites clustered together because they had less bare substrate and considerable cyanobacteria (*Lyngbya* sp.). Cellular diagnostics revealed that the stony coral *Porites astreoides* at all sites was stressed compared to colonies from a more remote Bahamian site. Offshore corals exhibited higher biomarker accumulations than inshore colonies. Corals near ocean outfalls and from protected areas in the Florida Keys had diagnostic profiles consistent with treated wastewater exposure. Profiles of offshore colonies were consistent with xenobiotic detoxification. Corals regenerated from sampling lesions at the two offshore biomonitoring control sites and two other inshore sites. Regeneration rates at offshore sites near the offshore ocean outfall and shipping channel were negative. Congruence between ecological, physiological and molecular information demonstrates that using multiple bioindicators can identify linkages between land-based sources of pollution and distressed coral reefs.

Key words: biomarkers, *Porites astreoides*, coral reef health, treated wastewater

Introduction

Land-based sources of pollution are critical factors influencing the fate of coral reefs off the heavily populated southeast Florida coast. Here, we describe a project that integrates traditional monitoring methods with new bioindicator technology – cellular diagnostics – to address how treated wastewater discharge and shipping channels affect coral reefs.

Cellular diagnostics was designed to assess the condition of reef-building corals and identify mechanisms of coral pathologies (Downs 2005 and references therein). It works because environmental stressors affect organisms by overwhelming defenses at lower levels of the biological hierarchy: molecular, cellular, and organismal-level homeostatic processes. By evaluating coral responses at these and higher levels of the biological hierarchy, scientists can provide resource managers with critical information needed to identify and ameliorate stressors before an ecosystem-scale crisis occurs (Fauth et al. 2003)

Methods

Sampling occurred at four paired, inshore and offshore stations located near a treated wastewater outfall, an inlet mouth, a treated wastewater outfall located within an inlet mouth, and a biomonitoring control site off Broward Country, Florida, USA (Table 1). The mustard hill coral (*Porites astreoides* Lamarck) was chosen as the focal species because it is distributed across the shelf in south Florida and is amenable to cellular-diagnostic analyses.

Cellular Diagnostics

Diagnostic antibodies included assays for oxidative stress (copper-zinc superoxide dismutase [Cu/Zn SOD] and ferrochelatase [FC]), cellular metabolic condition (glucose-regulated protein [GRP 75] and total small heat shock proteins [sHsp]), protein metabolic condition (ubiquitin and heat shock protein 60 [Hsp 60], indicators of xenobiotic response (cytochrome P450-2 class [CYP 2], cytochrome P450-6 class [CYP 6], cnidarian glutathione-S-transferase [GST] and multi-drug resistance protein [MDR]). Details are provided in Fauth et al. (2006).

Corals were sampled with a 1.5cm punch, placed in opaque canisters underwater, blotted dry on deck, frozen on dry ice, and stored at -80° C. Frozen samples were ground to a powder, proteins extracted

in buffer, repeatedly centrifuged to generate a soluble protein (TSP) and assayed according to methods described in Downs (2005).

Station	Ridge position	Depth (m)	Reference Location	Treatment
HWO2	Middle	9	Hollywood	Ocean outfall
HWO3	Outer	16	Hollywood	Ocean outfall
PE2	Middle	8	Port Everglades	Inlet/ship harbor
PE3	Outer	15	Port Everglades	Inlet/ship harbor
HI2	Inner	9	Hillsboro Inlet	Ocean outfall + inlet
HI3	Outer	16	Hillsboro Inlet	Ocean outfall + inlet
FTL1	Middle	9	Fort Lauderdale	Control
FTL3	Outer	17	Fort Lauderdale	Control

Table 1. Sampling sites (see Banks et al 2007 for precise location)

Coral Colony Health

Health of coral colonies was assessed by quantifying the rate at which hole-punch lesions healed (Fisher et al. 2007). Five colonies at each site were sampled in January, 2005, and re-measured 8 mos later. Lesion area was calculated as an ellipse, and regeneration rate expressed as $mm^2\ d^{-1}$. We also estimated percent old and recent tissue loss using Atlantic and Gulf Reef Rapid Assessment (AGRRA) protocols (Kramer et al. 2005).

Ecosystem Assessment

Digital video 15-25 m transects were used to estimate percent cover. Video was converted to single-frame for pointcounting (15 pts./frame) to estimate percent projected cover of uncolonized (bare) substrate, stony corals and other functional groups: gorgonians, zooanthids, porifera, macroalgae, and the cyanobacteria *Lyngbya* (Dustan et al. 1999).

Statistical Analyses

Multivariate analysis of variance tested the null hypothesis that reef location (inshore versus offshore), and proximity to treated wastewater outfalls and shipping channels (diagnostic responses transformed as $\log_{10}(x + 1)$ had no effect on coral responses . Separate univariate tests interpreted significant MANOVA results and Tukey's Honestly Significant Difference separated univariate means. Hierarchical clustering revealed patterns in coral diversity, bottom cover and cellular-diagnostic responses. All analyses used JMP V. 4.0.4 (SAS Institute, Inc., Cary, NC, USA) at $\alpha = 0.05$.

Results

Community Composition

Sites clustered into two groups. Three inshore sites (FTL1, PE2 and HWO2) had the lowest substrate cover, highest *Lyngbya* , and least cover by sponges, stony corals and soft corals. All four offshore sites plus the inshore HI2 site clustered together because they had the highest percentages of bare substrate, the lowest cover of *Lyngbya*, and the most cover by sponges, stony corals and soft corals. (Fig. 1).

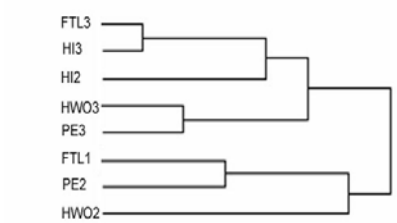

Fig 1. Sites clustered by community composition as percentage cover of functional groups described in text.

Cellular-Diagnostic Responses

Significant variation in the vector of ten cellular-diagnostic responses was explained by depth ($P < 0.001$), proximity to ocean outfalls ($P < 0.003$), depth x shipping channel ($P < 0.04$) and ocean outfall x shipping channel ($P < 0.02$). 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 with depth or proximity to potential land-based sources of pollution (Fig. 2).

Offshore corals, averaged across all sites, had significantly more Hsp 60 (protein metabolic condition) than did inshore corals ($P < 0.006$). Colonies from HI, a site near both a treated wastewater outfall and shipping channel, had less Hsp 60 than expected (significant interaction term: ANOVA $F_{1,29} = 10.57$, $P < 0.003$). Ubiquitin levels varied with the depth x shipping channel interaction ($P < 0.043$). Corals at inshore sites without a nearby shipping channel had twice as much ubiquitin as colonies at inshore sites with a shipping channel nearby. In contrast, corals at offshore sites had intermediate ubiquitin levels (Fig. 2)..

Grp 75 levels varied with the ocean outfall x shipping channel interaction ($P < 0.001$). Averaged across both depths, corals at HWO had more than three times as much Grp 75 as colonies at the biomonitoring and HI sites. FC levels varied significantly with the depth x ocean outfall interaction ($P < 0.03$). Corals at inshore sites near ocean outfalls had less FC than colonies at inshore sites without an ocean outfall and offshore sites with an ocean outfall nearby. Offshore colonies at sites without an ocean

outfall were indistinguishable statistically from these two groups (Fig. 2).

CYP 2 and GST levels were both higher offshore ($P < 0.02$). Averaged across depths, sites without shipping channels had CYP 6 levels 26% higher than sites with shipping channels ($P = 0.045$). Mean MDR levels varied with depth, ocean outfall, and the ocean outfall x shipping channel interaction (all $P < 0.034$). On average, mean MDR levels were 26% lower inshore compared to offshore sites, and 30% lower near ocean outfalls compared to those without them. In addition, mean MDR levels were significantly higher at the FTL biomonitoring sites than at the two HWO sites. Mean MDR levels at the paired PE and HI sites were statistically indistinguishable from both of these groups (Fig. 2).

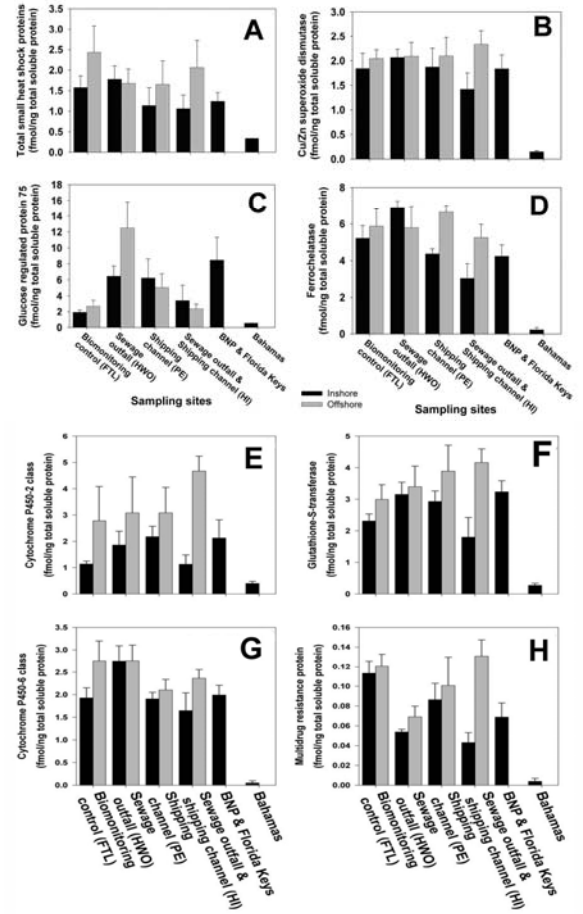

Fig. 2 Mean (± SE) accumulation levels for small heat shock proteins (A), Cu/Zn superoxide dismutase (B), glucose regulated protein 75 (C), ferrochelatase (D), Cytochrome P450-2 (E), glutathione-S-transferase (F), Cytochrome P450-6 class (G),multidrug resistance protein (H).

Coral Lesion Healing

Lesion regeneration rate 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

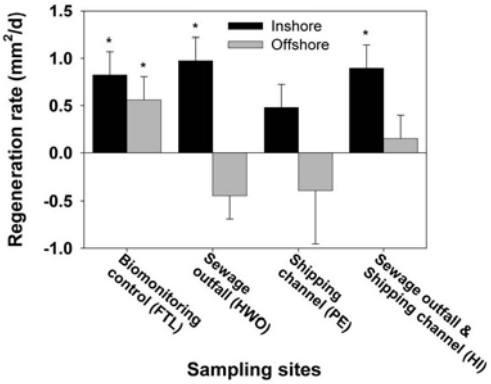

Fig 3. Regeneration rates (mm^2/d) of mustard hill corals (*Porites astreoides*). Data are least squares means (± 1 SE) from analysis of covariance, with initial lesion size as the covariate. Asterisks signify regeneration rates that differed significantly from zero. N = 1-5 colonies at each location.

significantly greater than zero (Fig. 3). In contrast, among the offshore sites only colonies at the biomonitoring control site had regeneration rates significantly greater than zero. 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. 3).

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, Downs et al. 2005). In *Porites astreoides* sampled off Broward County, percent tissue loss regressed positively on GRP 75 accumulation (regression equation: Log_{10}(% mortality + 1) = 0.48 + 0.042[GRP 75 + 1]; hereinafter, concentrations in fmol/ng total soluble protein; $P < 0.008$, $R^2 = 0.20$), which is essential for cell proliferation. Backward stepwise selection identified a log-log model with Grp 75, ubiquitin, CYP 2 and CYP 6 as predictors of percent tissue loss. Coral colonies with low ubiquitin levels and high levels of Grp 75, CYP 2, and especially CYP 6 lost the most tissue (regression equation: Log_{10}(% mortality + 1) = $1.32 + 0.042\log_{10}$[GRP 75 + 1] - $0.84\log_{10}$[ubiquitin + 1] + $064\log_{10}$[CYP 2 + 1] + $2.15\log_{10}$[CYP 6 + 1]; $P < 0.005$, $R^2 = 0.40$). Similarly, levels of ubiquitin, CYP 2 and CYP 6 were significant predictors of lesion regeneration rate (regression equation: Log_{10}(% mortality + 1) = $-0.091 + 1.03\log_{10}$[ubiquitin + 1] – $1.32\log_{10}$[CYP 2 + 1] - $3.14\log_{10}$[CYP 6 + 1]; $P < 0.005$, $R^2 = 0.40$). Coral colonies with high ubiquitin levels and low levels of CYP 2 and CYP 6 possessed highest regeneration rates (Figure 4).

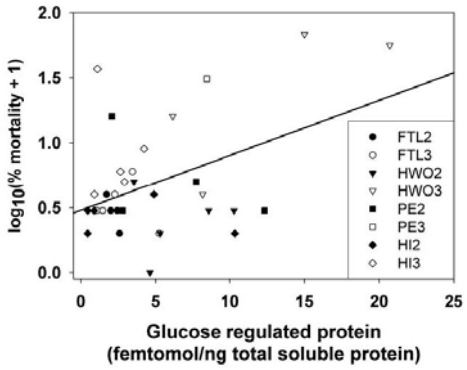

Fig 4. Log-linear regression of coral tissue mortality (%) as a function of glucose regulated protein (mortalin). Regression equation: Log 10 (mortality +1) = 0.48 + 0.042 Grp 75 (femtomoles/ng soluable protein). $F_{1,32}$= 8.10, P<0.008, R^2 = 0.20.

Comparisons with other Geographical Locations

Accumulations of every cellular-diagnostic parameter at the eight Broward County sites were indistinguishable from *P. astreoides* sampled the same month in Biscayne National Park and the Florida Keys. However, all biomarkers at every U.S. site were higher than those of *P. astreoides* from offshore Little Exuma Island, the Bahamas (two colonies, located approx. 23°30 N, 75°23W). The only exception was Hsp 60, which was undetectable in Bahamian corals and not differ from zero in inshore HI colonies. Cluster analysis (see Fauth et al. 2006) confirmed these analyses and the two Hollywood outfall sites (HWO2 and HWO3) clustered with FKNMS (Florida Keys National Marine Sanctuary) corals; the remaining inshore and offshore sites formed natural groups.

Discussion

Coral responses at the cellular, individual, and community levels provide a comprehensive assessment of the potential effects of land-based sources of pollution. Considered together, they suggest that corals off the southeastern Florida coast are exposed to poor-quality water, which reduces the ability of colonies to repair small lesions such as those created by our biopsy punches or by herbivores such as parrotfishes. In turn, this may reduce the ability of corals to colonize, grow and reproduce, thereby contributing to the low coral cover typical of this area (Banks et al. 2007). A study conducted further south in the Florida Reef Tract (Fisher et al. 2007) drew similar conclusions, which suggests this pattern may be widespread and common.

In our study, cellular diagnostic parameters indicative of xenobiotic stress were elevated at all our stations and in Biscayne National Park and Florida Keys National Marine Sanctuary, compared to corals in the Bahamas. This broad pattern illustrates the need to include a control distant from major sources of anthropogenic stress. *Porites astreoides* from the Bahamas had very low levels of all cellular-diagnostic parameters, and total sHsp was below detection limits there. 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 Hollywood treated wastewater outfall and in the FKNMS possessed high levels of GRP 75, or mortalin. This enzyme is induced by glucose deprivation and involved cellular senescence and transformation. 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 treated wastewater alters coral nutrition by generating higher than normal protein turnover rates , which inhibits coral growth and recruitment and results in decreased coral cover (Pastorak and Bilyard 1985, LaPointe et al. 2004).

Elevated levels of Hsp 60, CYP 2 and MDR at offshore sites FTL3, PE3 and HI3 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 conjugates the oxidized xenobiotic to glutathione, which is then pumped from the cell by multidrug resistance protein thus lowering 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.

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 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 at 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. 2005). Inshore habitats are intrinsically more variable and much of the difference between inshore and offshore corals may have an ecotypic basis.

Total tissue loss was greatest near the City of Hollywood's treated wastewater outfall and moderately high at the offshore Port Everglades and Hillsborough Inlet stations. These three stations also had lesion regeneration rates indistinguishable from zero, and in two cases (HWO3 and PE3) tended to be negative; lesions grew larger instead of healing. Inability to regenerate small lesions indicates that conditions were poor for coral growth and reproduction at these sites between January and August, 2005.

Greatly elevated levels of cellular-diagnostic parameters and low coral cover at all sites we sampled off Broward County are cause for concern, especially because regeneration rates were indistinguishable from zero at four of eight stations. Our study establishes a line of evidence suggesting that land-based sources of pollution negatively affected the status and trends of these coral reef communities, which therefore should receive greater protection from potential damage caused by these stressors.

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