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Cellular diagnostics and coral health: Declining coral health in the Florida Keys

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Abstract

Coral reefs within the Florida Keys are disappearing at an alarming rate. Coral cover in the Florida Keys National Marine Sanctuary declined by 38% from 1996 to 2000. In 2000, populations of *Montastraea annularis* at four sites near Molasses Reef within the Florida Keys National Marine Sanctuary and one reef within Biscayne National Park were sampled on a quarterly basis. Anecdotal observations showed corals at Alina's Reef in Biscayne National Park appeared healthy in March, but experienced an acute loss of coral cover by August. Cellular Diagnostic analysis indicated that Alina's Reef corals were in distress: they had been afflicted with a severe oxidative damaging and protein-denaturing stress that affected both the corals and their symbiotic zooxanthellae. This condition was associated with a significant xenobiotic detoxification response in both species, reflecting probable chemical contaminant exposure. These results demonstrate that applying a Cellular Diagnostic approach can be effective in helping to identify stress and its underlying causes, providing diagnostic and prognostic biomarkers of coral health.

Keywords: Biomarker; Cellular diagnostics; Coral; Florida Keys; Coral health

1. Introduction

Coral reefs are experiencing rapid declines from deteriorating environmental conditions across the globe (Dustan and Halas, 1987; Bryant and Burke, 1998; Dustan, 1999; Hoegh-Guldberg, 1999; Wilkinson, 1999; Gardner et al., 2003). Recent reports indicate that 58–70% of coral reefs worldwide are directly threatened by human-associated activities, while over 80% of the Caribbean coral-reef cover has disappeared in the last 30 years (Bryant and Burke, 1998; Wilkinson, 1999; Hoegh-Guldberg, 1999; Goreau et al., 2000; Gardner et al., 2003). Coral reef communities experiencing persistent environmental disturbances (e.g., coastal development and land-based pollution) are undergoing changes that lead to a loss of coral diversity, increased incidence of disease, reduced growth, reduced reproduction, and mass mortality (Richmond, 1993; Hoegh-Guldberg, 1999; Nystrom et al., 2000; Knowlton, 2001; Porter and Tougas, 2001; Patterson et al., 2002; CRMP, 2001). From 1996 to 2000, a time span that included a major El Niño event, corals in the Florida Keys National Marine Sanctuary lost a record 38% of their living coral cover (CRMP, 2001; Porter et al., 2001;

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Turgeon et al., 2002). The causes of these devastating declines, however, remain largely unknown.

The primary question facing resource managers is whether coral declines result from climatic forces, local or regional anthropogenic factors, or the synergistic effects of these different forces. If declines are caused solely by global factors, there may be little resource managers can do to immediately ameliorate the problem. However, if local (defined as a restricted region of about 50 km) factors contribute to coral declines, resource managers may be able to respond with mitigation efforts. For example, if urban effluent is the principle stressor, discharges can be managed and regulated. Unfortunately, conventional coral monitoring and mapping protocols have had limited success in demonstrating the causes of coral reef declines in the Florida Keys and elsewhere (Pennisi, 2002; Vasseur and Cossu-Leguille, 2003). Most monitoring programs were designed to establish baseline conditions and determine trends, not to identify sources of stress (Risk, 1999). The inability of coral monitoring programs to pinpoint factors affecting local reef declines makes it difficult for resource managers to effectively manage coral reefs (Jameson et al., 2001, 2002; Downs et al., 2005).

Resource management can be more effective if a coordinated, interdisciplinary (e.g., biochemistry, biology, chemistry, geology, and toxicology) approach is used to rapidly diagnose population health and accurately forecast expected outcomes of different mitigation strategies (Risk, 1999; Vasseur and Cossu-Leguille, 2003). We recently developed a novel biotechnology that employs a suite of diagnostic indicators to assess the cellular-physiological condition of reef-building corals (Downs et al., 2000, 2002a,b; Brown et al., 2002a; Woodley et al., 2002; Fauth et al., 2003; Downs, 2005). Cellular Diagnostics focuses a comprehensive array of biotechnologies to diagnose population health, and was tailored to determine coral-health condition and help identify mechanisms of coral pathologies (Downs et al., 2000, 2002a; Brown et al., 2002a; Woodley et al., 2002; Table 1). Cellular Diagnostics is used to measure changes in cellular parameters, allowing: (1) assessment of cellular-physiological condition of an individual or population, (2) identification of putative stressors, either by direct measurement of the stressor or by profiling stressor-specific effects, and (3) forecasting higher-order behavior based on an understanding of cellular-level processes (Downs, 2005). Parameters that are measured as part of a cellular diagnosis include membrane integrity and composition, anti-oxidant redox status, protein metabolic condition, xenobiotic detoxification pathways, intra-cellular metal regulation, and genomic integrity. Cellular Diagnostics provides a new approach to environmental-health assessment, based on concepts and methodologies already used in medical diagnostics and epidemiology (Downs, 2005).

Previously, we used this Cellular Diagnostics approach to demonstrate that oxidative stress was a factor in the pathology of seasonal coral bleaching (Downs et al., 2000, 2002a; Brown et al., 2002a,b). Here, we assess the health of corals at five sites near Molasses Reef within the Florida Keys National Marine Sanctuary during a non-El Niño/La Niña climatic year (2000) and an additional reef located at some distance from these sites within another protected area: Biscayne National Park. Our objectives were to: (1) document baseline, seasonal changes in cellular parameters during a non-elevated sea-surface temperature period, (2) provide an interpretive diagnosis of the health condition of these corals in 2000, and (3) determine whether stressful factors affecting corals in the Florida Keys could include local sources (e.g., agriculture and urban run-off, high-boat density, etc.) in addition to known climatic forces such as El Niño/La Niña phenomena.

2. Materials and methods

2.1. Reagents

All chemicals for buffered solutions were obtained from Sigma Chemicals Co. (St. Louis, Missouri, USA) or EnVirtue Biotechnologies, Inc. (Winchester, VA, USA). PVDF membrane was obtained from Millipore Corp. (Bedford, Massachusetts, USA). Antibodies against all the cellular parameters, as well as calibrant standards, were obtained from EnVirtue Biotechnologies, Inc. (Winchester, VA, USA). Antibodies were raised against an 8–12 residue polypeptide conjugated to ova albumin. Antigens were designed based on extremely conserved and unique domains found within the target protein. Rabbits were immunized with the antigen with a Ribi-adjuvant carrier. All antibodies used in this study were immuno-purified with a Pierce SulfoLink Kit (cat.# 44895) using the original unconjugated peptide as the affinity binding agent. Anti-rabbit conjugated horseradish peroxidase antibodies were obtained from Jackson Immunoresearch (West Grove, PA, USA).

2.2. Coral collection

We sampled boulder corals (Montastraea annularis species complex) at four sites near Molasses Reef, within the Florida Keys National Marine Sanctuary (FKNMS), Key Largo, FL, USA (Fig. 1). These sites were sampled in 1999 within a previous study (Downs et al., 2002a), when a bathymetric gradient in bleaching was observed; in 1999, fewer colonies bleached in shallower (3 and 6 m sites) than in deeper water (9 and 18 m sites; Downs et al., 2002a). Site 1 is designated as the 3 m site; site 2 is designated as the 6 m site; site 3 is designated as the 9 m site and site 4 is designated as the 18 m site. We also

Table 1
Cellular biomarkers and their physiological functions

Cellular parameter	Biological significance
Manganese superoxide dismutase	(MnSOD) Manganese SOD is localized to the mitochondria in eukaryotic cells, both in algae and in cnidarians. Superoxide dismutases accumulate in response to oxidative stress and are one of the main anti-oxidant defense pathways. Superoxide dismutases are an index that the cell is responding to an oxidative stress, and more so, that Mn SOD is a specific index that the mitochondria are experiencing an oxidative stress
Glutathione peroxidase	(GPx) Glutathione peroxidase is an important enzyme in cellular antioxidant defense systems. This selenoprotein detoxifies hydroperoxides and organic peroxides, including the peroxides of free (but not esterified) fatty acids and other lipids, by catalyzing the reaction, $ROOH + 2GSH \rightarrow GSSG + ROH + H_2O$ The majority of this enzyme activity (60–75%) is found in the cytoplasm of eukaryotic cells, and the remaining activity (25–40%) is in the mitochondria. GPx is inducible in response to oxidative stress
Glutathione-s-transferase	(GST) The name glutathione transferase refers to only one of several enzymatic activities that these enzymes possess. Other activities include glutathione-dependent isomerase activity, which is important in prostaglandin biosynthesis, and selenium-independent glutathione peroxidase activity (alpha and theta subclasses of GST). Glutathione transferases are usually associated with detoxification by conjugation of glutathione to genotoxic and cytotoxic xenobiotic electrophiles derived from drugs, carcinogens, and environmental pollutants. During a xenobiotic challenge, glutathione can be conjugated to a xenobiotic by GST, representing a major detoxification pathway (Sies, 1999). Additionally, GST detoxifies DNA hydroperoxides, playing an important role in DNA repair. GST also acts as one of the main defenses for detoxifying 4-hydroxynonenal in the cell (DeZwart et al., 1999)
P-glycoproteins 160 and 180	(MDR or MXR—multixenobiotic resistance protein) P-glycoprotein plays a role in xenobiotic detoxification. Currently, it is believed that P-glycoprotein effectively processes certain xenobiotics and cellular toxins to exit the cell. Sustained exposure to certain xenobiotics causes an increase in the cellular level of P-glycoproteins. P-glycoproteins are members of a superfamily of proteins called the adenosine triphosphate binding cassette that act as channels and transporters of solutes across membranes. Induction of certain P-glycoproteins indicates a response to xenobiotic exposure
Heat-shock protein 60 and 70	(Hsp60 and Hsp70) Hsp60 and Hsp70 are molecular chaperonins. Chaperones regulate protein structure and function under normal physiological conditions as well as during and following stress by renaturing denatured proteins into active states in an ATP-dependent manner. Both Hsp60 and Hsp70 are ubiquitous chaperonins found in all phyla of life and are essential components for cellular function, during both normal and stressed conditions. Furthermore, mitochondrial and chloroplastic Hsp60 and Hsp70 homologues work in concert—an essential multi-step pathway for correct conformation of protein structure. Hsp60 and Hsp70 levels increase in response to stress, specifically in response to increased protein synthesis and denaturation
Invertebrate small heat-shock proteins	(sHsp = total small heat-shock protein isoforms)—αB-crystallin, Hsp22, Hsp23, Hsp26, and Hsp28 share domains of common homology to one another, but have different cellular functions. In cnidarians, as many as 5–6 major sHsp isoforms exist (Downs et al., 2000). Small Hsps from all phyla share a common motif near the carboxyl-terminal end of the protein, known as the "heat-shock domain" or α-crystallin domain. Other areas of these proteins are not homologous and are specific to the sub-family of sHsps. In most cases, the small heat-shock proteins are not present during optimal growing conditions and are only elicited by stress. αB-crystallin is a small heat-shock protein found only in the cytosol of animals, where it protects cytoskeletal elements during stress (Derham and Harding, 1999). Thus, the presence and concentration of different small heat-shock proteins reflects the physiological status of several metabolic and structural pathways in the cell
Chloroplast small heat-shock protein	(Chlp sHsp) A member of the small heat-shock protein family found only in plants. This protein functions to protect Photosystem II activity, specifically the oxygen evolving complex, during photooxidative conditions. This protein is not normally present during non-stressed conditions, but is induced by heat stress, high-light stress, UV stress, and during exposure to some herbicides (e.g., atrazine)
Ubiquitin	Ubiquitin is a 76-residue protein found in most phyla of life and used in a process for marking proteins for rapid degradation. Ubiquinated proteins are degraded by proteolytic enzymes known as proteosomes. Proteins, during stress, are targeted for degradation usually because these proteins have undergone an irreversible denaturation. Increases in ubiquitin levels are an indication of increased levels of protein degradation, and hence, increased protein turnover. Consequently, to compensate for decreased functional protein levels due to stress, the cell will increase production of these same proteins. Thus, measurement of levels of ubiquitin is an index of the structural integrity of the protein component of the superstructure of the cell. Increased ubiquitin levels indicates: (1) a protein denaturing stress is occurring; (2) increased expenditure of energy is required to compensate for this stress-induced protein turnover; and (3) in comparison to baseline data of this parameter for a particular species, may act as an indicator of individual fitness

sampled one site at Alina's Reef, within Biscayne National Park (BNP), Homestead, FL, USA (site 5). We included it as a northern, shallow (6 m) site, potentially influenced by agricultural and landfill runoff and operations of two pressured-water nuclear reactors at Turkey Point.

We collected samples (2.5 cm diameter) from the same five colonies at each site in March, June, August,

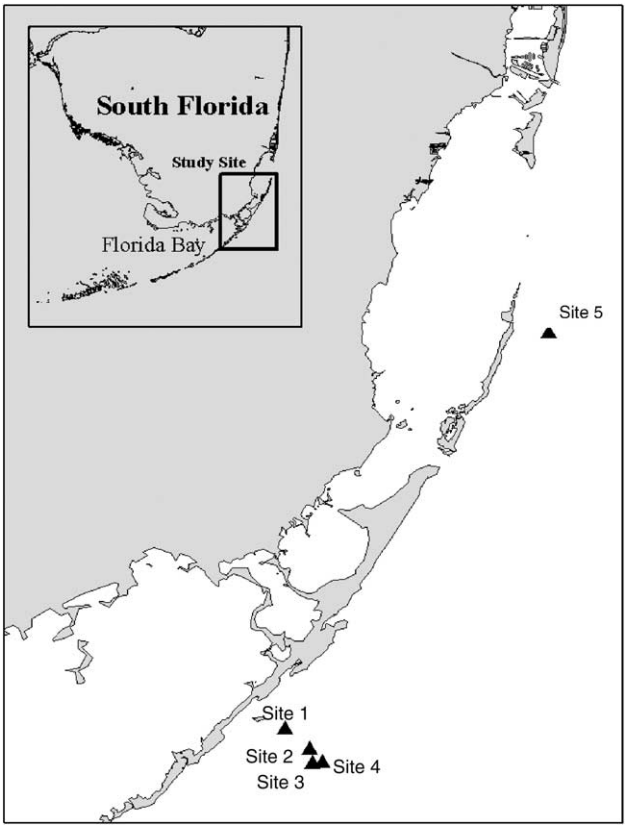

Fig. 1. Map of sampling region in the Florida Keys, USA. Site 1 is Rodriguez Key, 3 m depth. Site 2 is southwest of Three Sisters, 6 m depth. Site 3 lies between Molasses Reef and Pickles Reef, 9.1 m depth. Site 4 is southwest of Molasses Reef, 18 m depth. Site 5 is Alina's Reef which lies within Biscayne National Park, 6 m depth.

and November 2000. Individual colonies in the Florida Keys were identified and tagged in 1999, while colonies in Alina's Reef were identified and tagged in March 2000. Throughout the year, the same colonies were re-sampled at each of the sampling time points, with the exception of August and November at Alina's Reef as the result of whole colony mortality. In August and November, new coral colonies were sampled at Alina's reef to compensate for the coral colonies that died. Samples were biopsied using a modified leather punch, and then placed in black film canisters. On deck, water was quickly removed from the film canister and samples transferred to a liquid nitrogen dry shipper. Samples were stored at −8 °C until analysis.

2.3. Sample preparation, ELISA validation, and ELISA

Coral samples were ground to a powder using a liquid nitrogen-chilled ceramic pestle and mortar. Samples (~10 mg) of frozen tissue were placed in 1.8 ml microcentrifuge tubes along with 1400 μl of a denaturing buffer consisting of 2% SDS, 50 mM Tris–HCl (pH 6.8), 25 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, 0.01 mM AEBSF, 0.04 mM Bestatin, 0.001 E-64, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 0.01 mM apoprotin, 5 μM a-amino-caproic acid, and 1 μg/100 ul pepstatin A. Samples were heated at 95 °C for 3 min, vortexed for 20 s, incubated at 95 °C for another 3 min, and then incubated at 25 °C for 5 min. Samples were centrifuged at $10{,}000 \times g$ for 10 min. Supernatant free of a lipid/glycoprotein mucilage matrix was transferred to a new tube, centrifuged at $10{,}000 \times g$ for 5 min and supernatant free of a lipid/glycoprotein mucilage matrix was again transferred to a new tube and subjected to a protein concentration assay (Ghosh et al., 1988).

To ensure equal sample loading, 40 μg of total soluble protein of samples from one site and time point were loaded onto a 12.5% SDS-PAGE gel (16 cm), the gel was run until the bromophenol blue dye front was near the bottom of the gel, stained with a Coomassie blue solution (BB R-250) overnight, and then destained for 4 h with multiple washes of destaining solution. Equal loading was determined by visualization and optical density using a Canonscan scanner and analysis performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The purpose of this method is to visually validate the protein concentration assay and to ensure that sample artifact has not occurred 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 micrograms of total soluble protein of coral supernatant was loaded onto a 20-, 16-, or 8-cm SDS-polyacrylamide gel with various concentrations of bis/acrylamide. A Tri(2-carboxyethyl)phosphine (TCEP) concentration of 1 mM was added to gels loaded with samples to be assayed with antibody to the chloroplast sHsp and the invertebrate sHsp. Tri(2-carboxyethyl)phosphine is a reductant that can be used in acrylamide gels without interfering with acrylamide polymerization. Proteins can often spontaneously form disulfide bonds in the loading buffer, in the stacker gel, and in the separating gel, causing homomeric or heterometic dimerization. Proteins (e.g., small heat-shock proteins) whose very nature are to dimerize under adverse environmental conditions will readily aggregate under standard SDS-PAGE procedures. Gels were blotted onto PVDF membrane using a wet transfer system. Membrane was blocked in 7% non-fat dry milk, and incubated with the primary antibody for 1 h. The blots were washed in tris-buffered saline (TBS)-0.05% Tween (v/v) four times, and incubated

in a horseradish peroxidase-conjugated secondary antibody solution for 1 h. Blots were washed four times in TBS, and developed using a chemiluminescent reporter system.

Once validated, antibodies and samples were optimized for ELISA using an $8 \times 6 \times 4$ factorial design (Crowther, 1999). Every ELISA assay must be optimized for the proper concentration of protein loading, antibody titer, sample-to-standard calibration, and handling procedures as a measure of quality control and quality assurance (Downs, 2005). A Beckman-Coulter Biomek 2000 using 384-well microplates was used to conduct the ELISA assays. Samples were assayed with the following EnVirtue Biotechnologies, Inc. antibodies (generated in rabbits): anti-algal glutathione peroxidase (GPx) (AB-G101-P), anti-algal manganese superoxide dismutase (MnSOD) (AB-S100-P), anti-algal heat shock protein 60 (Hsp 60) (AB-H100-P), anti-algal heat-shock protein 70 (Hsp 70) (AB-H101-P), anti-algal glutathione-s-transferase (GST) (AB-GST-PA), anti-chloroplast small heat-shock protein (chlp sHsp) (AB-H104-C), anti-ubiquitin (AB-U100), anti-cnidarian heat-shock protein 70 (Hsp 70) (AB-H101-CDN), anti-cnidarian heat-shock protein 60 (Hsp 60) (AB-H100-IN), anti-cnidarian manganese superoxide dismutase (MnSOD) (AB-S100-MM), anti-cnidarian glutathione peroxidase (GPx) (AB-GPX-IN), anti-cnidarian glutathione-s-transferase (GST) (pi-isoform homologue; AB-GST-INV), anti-invertebrate small heat shock protein (sHsp) (AB-H103), and anti-MDR (ABC family of proteins; P-glycoprotein 140 and 160; AB-MDR-160). Samples were assayed in triplicate with intra-specific variation of less than 6% for the whole plate. An eight-point calibrant curve using a calibrant relevant to each antibody was plated in triplicate for each plate.

2.4. Diagnostic strategy and statistical analysis

Planned (a priori) and unplanned comparison tests were used, depending on whether the question posed for a specific data set contained fundamental hypotheses, as in the case of a quantitative diagnostic strategy used by the Cellular Diagnostic System (Schaeffer, 1996; Panzer et al., 1999; Downs, 2005). Cellular Diagnostics posits that changes in cellular integrity and homeostatic responses are fundamental end-points in discriminating whether a xenobiotic or environmental agent is affecting the cell, hence the organism. The terms cellular integrity and homeostatic responses can be partially and stipulatively defined by the behavior of the parameters in the following categories: genomic integrity, protein metabolic condition, xenobiotic response, metabolic integrity, and oxidative-stress effects and response. It should be noted that these *definiens* of cellular integrity and homeostatic responses are used because they are measurable by the present technology. As the technology evolves, so will the categories and parameters; thus providing a more meaningful explanation for *cellular integrity* and *homeostatic response*.

Each category is defined by a functional process and the physical components constituting that process. For example, protein metabolic condition can be defined, in part, by the protein, RNA, and enzymatic components (sub-systems) that have a role in protein synthesis, protein maturation, and protein degradation. Shifts in these parameters are indicative of a shift in the equilibrium of these sub-systems, as well as the system as a whole. The behavior of each cellular parameter is independently regulated from the behavior of other cellular parameters, but can be affected by the behavior of other systems.

Parametric statistics were used to test whether cellular parameters differed among sites at the same time point and at different time points. If data were normally distributed and homogeneous, a one-way analysis of variance (ANOVA) with $\alpha = 0.05$ was employed. When Bartlett's test for homoscedasticity or graphical assessment of normality showed variates that did not meet ANOVA assumptions, we substituted a Welch ANOVA (Sokal and Rohlf, 1995). When significant differences were found among treatment means, we used the Tukey–Kramer honestly significant difference (HSD) method as an exact alpha-level test for all differences between means (Sokal and Rohlf, 1995).

3. Results

3.1. Antibody validation

Antibodies against cnidarian cellular parameters did not exhibit significant non-specific cross-reactivity (Fig. 2, Panels A–E), hence could be validly used in an ELISA format.

Antibodies against dinoflagellate cellular parameters also did not exhibit significant non-specific cross-reactivity (Fig. 3, Panels A–G). All blots are undoctored, full length documentation of the western blot. In Fig. 2A and D and Fig. 3C there is an artifact band at the top or bottom of the lane which are shadows from polyacrylamide gel edges, which is sometimes a common artifact of electro-transfer. In Fig. 2A, multiple bands are seen at a discreet migration rate. Glutathione peroxidase alpha is reknown for having multiple homologues. The antibody was raised against a conserved domain that is found in all homologues of GPx alpha, hence these homologues are detected in the blot. In Fig. 2B, a doublet band is seen for cnidarian GST. In high resolution gels, this can often be seen as a result of glutathione conjugation to the selenocysteine, thereby changing the GSTs migration rate. In Fig. 2E, the doublet band is the result of the expression of Hsp70 homologues, which

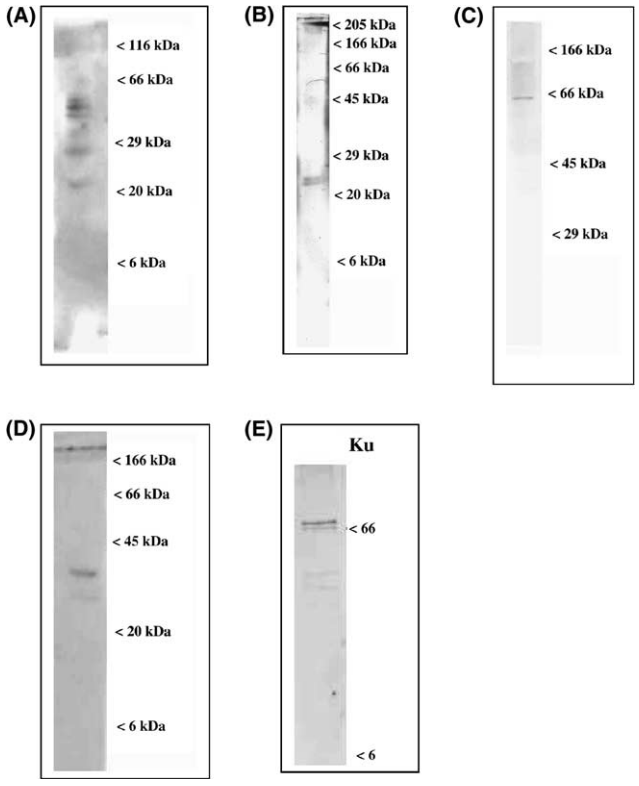

Fig. 2. Four random samples of *Montastraea annularis* were pooled, subject to SDS-PAGE, western blotted, and assayed with a specific antibody. (A) Antibody against cnidiarian GPx. (B) Antibody against cnidarian GST. (C) Antibody against cnidarian Hsp60. (D) Antibody against cnidarian MnSOD. (E) Antibody against cnidarian Hsp70.

are differentially regulated (Downs, unpublished data; C. Smith, personal communication). In Fig. 3A, a doublet band is seen for the chloroplast sHsp, which is the result of a 5–15 amino-acid residue homologue (Downs et al., 1999). Data for ubiquitin is not shown, because a western blot of ubiquitin is a smear, which is the result of single ubiquitin and the single and poly-ubiquitination of histones and proteins slated for degradation (Downs et al., 2000). A band was not detectable in the western blot for the invertebrate sHsp, but this is not unusual since only a severely stressed coral will exhibit this protein at a western blot detectable level (Downs et al., 2000).

Because of the evolutionary conservation of ubiquitin and MDR, these antibodies detected proteins from both species of the symbiosis in the coral homogenate (data not shown). Therefore, an ELISA measurement using this antibody detects total concentration for these proteins. In MDR, the faster migrating band is the dinoflagellate homologue while the slower migrating band is the cnidarian homologue. This was determined by running cultured Symbiodinium protein next to the coral homogenate using an SDS-PAGE/western blotting protocol (data not shown).

3.2. ELISA results

ELISA results are divided into the following Environmental Cellular Diagnostic System Categories:

- Protein Metabolic Condition which includes dinoflagellate Hsp60, dinoflagellate Hsp70, cnidarian Hsp60, cnidarian Hsp70 and Ubiquitin (Fig. 4),
- Metabolic Condition which includes the Chloroplast sHsp and the four major cnidarian sHsp classes (Fig. 5),
- Oxidative Stress Response which includes dinoflagellate MnSOD, dinoflagellate GPx, cnidarian MnSOD, cnidarian GPx (Fig. 6) and
- Xenobiotic Response which includes dinoflagellate GST, cnidarian GST and MDR (Fig. 7).

Subjective observations suggested little or no change in coral cover at sites 1–4 since our previous study in 1999 (Downs et al., 2002a). At Alina's Reef, coral coverage appeared unchanged from December 1999 to June 2000. By August, a clearly observable loss in coral cover occurred on the massive *Montastraea* heads at this site. Several coral heads that spanned more than 1 m in length were completely lost. There was significant coverage of brown algae on the dead coral, and it was therefore not possible to repeatedly sample one entire coral colony in August, and another one by November October.

The most striking biomarker pattern was exhibited by corals from Alina's Reef in March 2000. Parameters of protein metabolic condition suggested that a massive insult was occurring, and that the insult not only resulted in increased protein denaturation, but a considerable level of damage to functioning enzymes (Fig. 4, Table 1). Cnidarian and dinoflagellate Hsp70s and Hsp60s at Alina's Reef in March were significantly higher than Sites 1–4 at any time point (one-way ANOVA, $p < 0.05$; Fig. 4. Ubiquitin at Alina's Reef in March was considerably higher than almost all the sites at any time (one-way ANOVA, $p < 0.001$). Two conclusions that can be inferred from this data are (1) that the stressor was affecting only Alina's Reef and (2) that the stressor was also adversely affecting both Cnidarian and algal compartments of the coral symbiosis. Cnidarian and dinoflagellate Hsp70 and cnidarian Hsp60 remained high through November (one-way ANOVA, $p < 0.05$). Levels of dinoflagellate Hsp60 were no longer significantly elevated in August (ANOVA, $p < 0.01$), suggesting that at least organellar protein metabolism was returning to basal levels. Increased cnidarian Hsp60 suggests that mitochondrial function in the host remained perturbed through November.

Small heat-shock proteins protect various metabolic pathways and cellular function and usually are only present when these systems are experiencing a significant

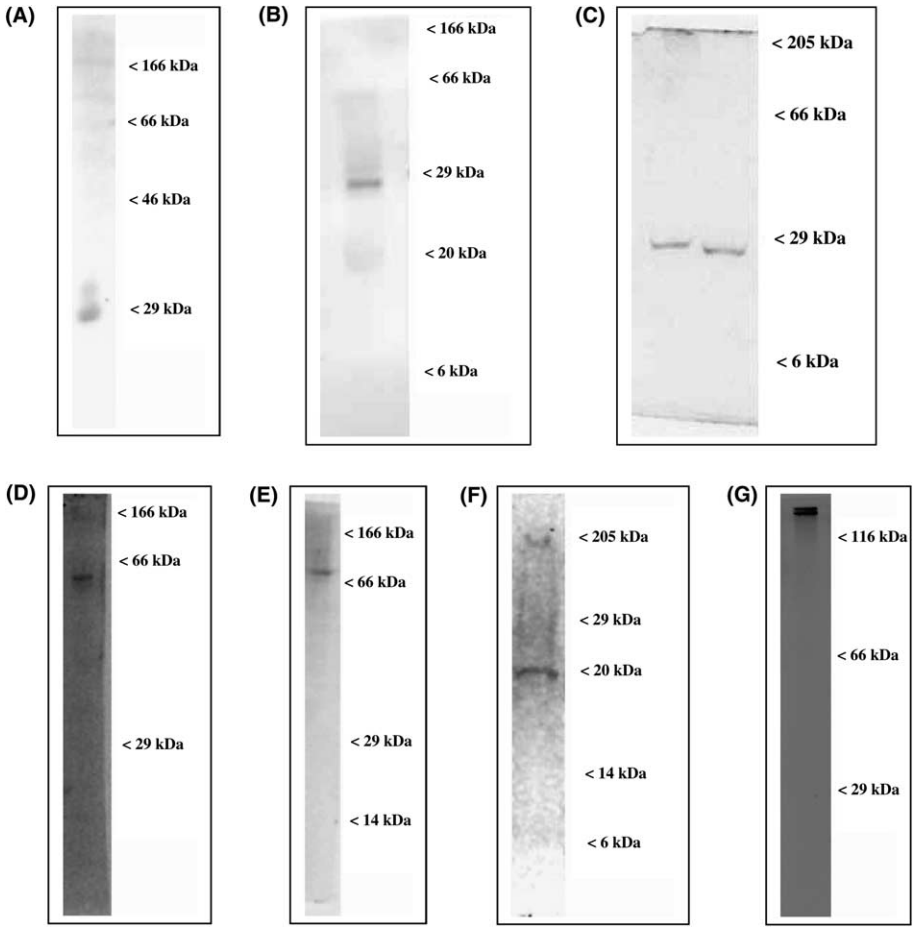

Fig. 3. Four random samples of *Montastrea annularis* were pooled, subject to SDS-PAGE, western blotted, and assayed with a specific antibody. In (C), individual samples were loaded onto the same gel in separate lanes to show sample consistency. (A) Antibody to the chloroplast small heat-shock protein. (B) Antibody to zooxanthallae GPx. (C) Antibody to zooxanthallae GST. (D) Antibody to zooxanthallae Hsp60. (E) Antibody to zooxanthallae Hsp70. (F) Antibody to zooxanthallae MnSOD. (G) Antibody that reacts with both zooxanthallae and cnidarian MDRs.

level of damage or dysfunction (Table 1). The chloroplast sHsp is an evolutionarily conserved protein found only in the chloroplast of photosynthetic eukaryotic organisms and protects Photosystem II of the photosynthetic electron-transport chain during stress (Heckathorn et al., 1998; Downs et al., 1999). Basal levels of this protein through the year in corals from sites 1–4 near Molasses Reef averaged 0.075 EU/ng TSP (Fig. 5). Levels of this protein at Alina's Reef in March were over twice this (Welch ANOVA, $p < 0.001$), indicating that PSII was severely stressed, if not damaged. Chloroplast sHsp levels then decreased to basal levels in June and remained there for the remainder of the year. Cnidarian sHsps had a similar pattern (Fig. 5), with cnidarian sHsp levels at Alina's Reef in March almost double the levels at the other sites through the year (Welch ANOVA, $p < 0.001$), but then decreasing significantly in June to levels comparable to seen at the other sites. The sHsp22 is localized to the mitochondria in cnidarians and other invertebrates and is thought to protect aspects of respiration, specifically oxidative phosphorylation (Morrow et al., 2000). These data suggest that the stressor(s) affected electron-transport of major metabolic pathways and destabilized cnidarian mitochondrial function for the remainder of the year.

Induction of both the chloroplast sHsp and the invertebrate sHsps suggests that oxidative damage may be occurring in both symbionts. To determine if antioxidant defenses of both species were elevated, we examined superoxide dismutases and glutathione peroxidases. Cnidarian MnSOD for corals sampled at Alinas Reef was significantly elevated throughout the year compared to sites 1–4 at all time points, but especially in March (Welch ANOVA, $p < 0.001$; Fig. 6). The expression pattern of dinoflagellate MnSOD reflected the pattern of cnidarian MnSOD, but its accumulation was even more pronounced in Alina's Reef, compared to the other sites at any of the time points (Welch ANOVA, $p < 0.001$). Elevation of both MnSODs suggests that the unknown stressor is affecting the mitochondria in both species. Both cnidarian and dinoflagellate GPx levels were significantly increased in March at

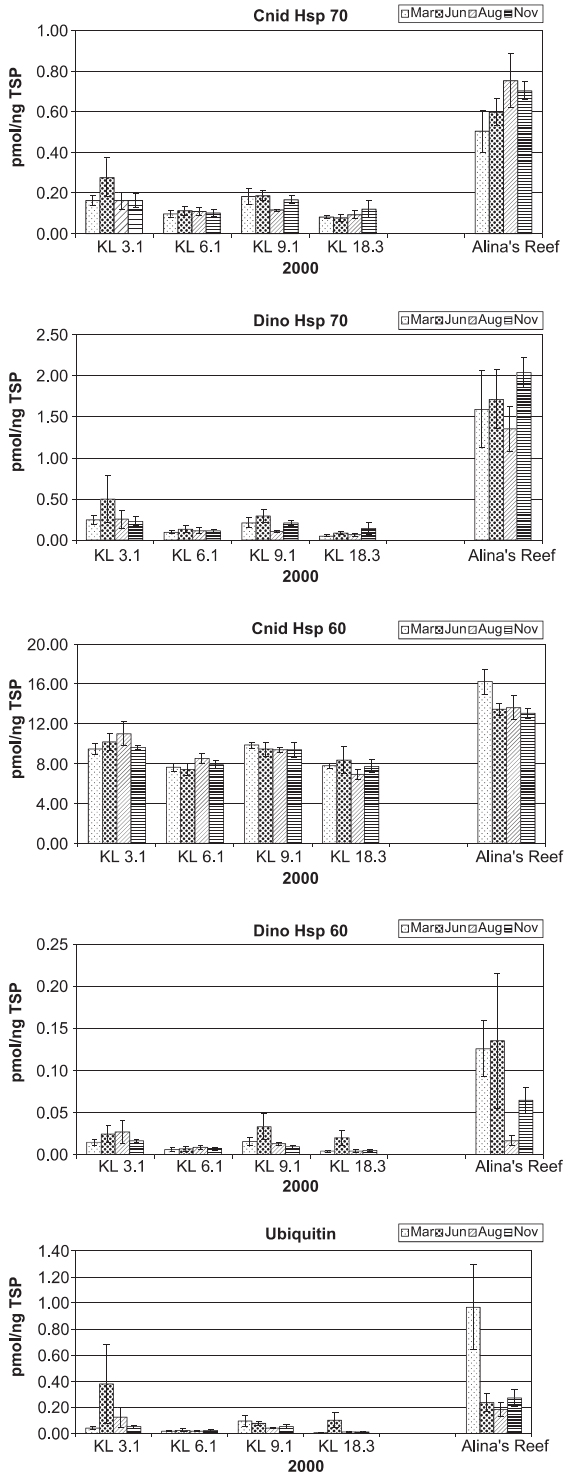

Fig. 4. Protein Metabolic Condition Biomarkers. Levels of cnidarian (Cnid) and dinoflagellate (Dino) biomarkers associated with Protein Metabolic Condition from all five coral reef sites that were sampled quarterly. Bars show untransformed means (±SE), $N = 5$ corals per site/date. Time point of sampling at each site from left to right: March, June, August, and November.

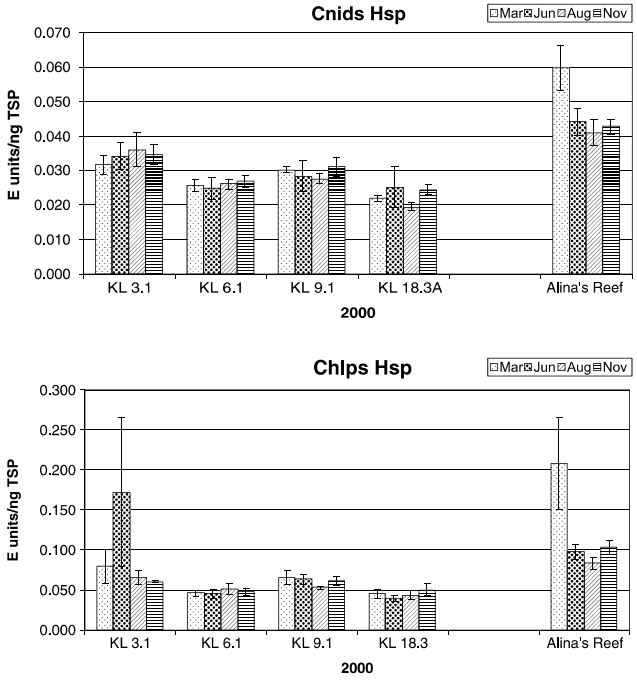

Fig. 5. Metabolic Condition. Levels of cnidarian (Cnid) and dinoflagellate (Dino) biomarkers associated with Metabolic Condition from all five coral reef sites that were sampled quarterly. Bars show untransformed means (±SE), $N = 5$ corals per site /date. Time point of sampling at each site from left to right: March, June, August, and November.

Alina's Reef (ANOVA, $p > 0.001$) but, as in the case of the MnSODs, decreased in accumulation in June through November, although these were still higher than GPx levels at Sites 1–4 ($p < 0.001$), Fig. 6. This increase of the major enzymatic anti-oxidant defenses suggests indicates that Alina's Reef was responding to an oxidative stress.

Combined results for parameters of Protein Metabolic Condition, small Hsps, and oxidative stress defense argue that Alina's Reef was experiencing a significant stress that was severely damaging proteins and threatening nominal metabolic equilibrium. Assays that examine mitochondrial membrane potential, mitochondrial electron transport, cytoskeletal integrity, and protein, lipid, and DNA oxidation would better refine the mechanism of cellular disruption that occurred at Alina's Reef. Some of these assays could be done in the field on the same day of collection, enhancing the ability of managers/scientists to identify and possibly mitigate the stressor.

The significant effect of the unknown stressor on both biomarker responses and coral cover at Alina's Reef strongly argues that the stressor is localized; at least localized to or around a region that includes Alina's Reef, but not Sites 1–4. Based on the proximity of Alina's Reef to a number of anthropogenic activities (e.g. nuclear power plant, South Florida Water Management Canals, agricultural runoff, Dade County Landfill, City of Miami) we hypothesised that the localized stressor

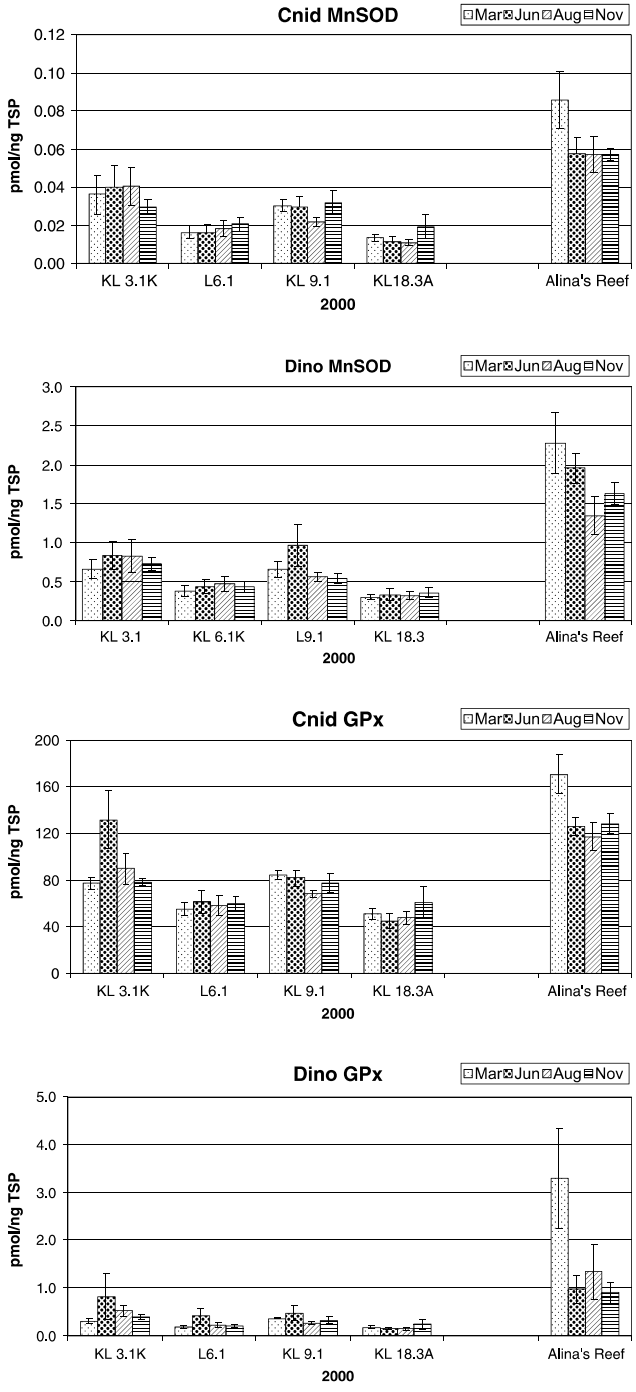

Fig. 6. Oxidative Stress Response. Levels of cnidarian (Cnid) and dinoflagellate (Dino) biomarkers associated with Oxidative Stress Response from all five coral reef sites that were sampled quarterly. Bars show untransformed means (±SE), N = five corals per site/date. Time point of sampling at each site from left to right: March, June, August, and November.

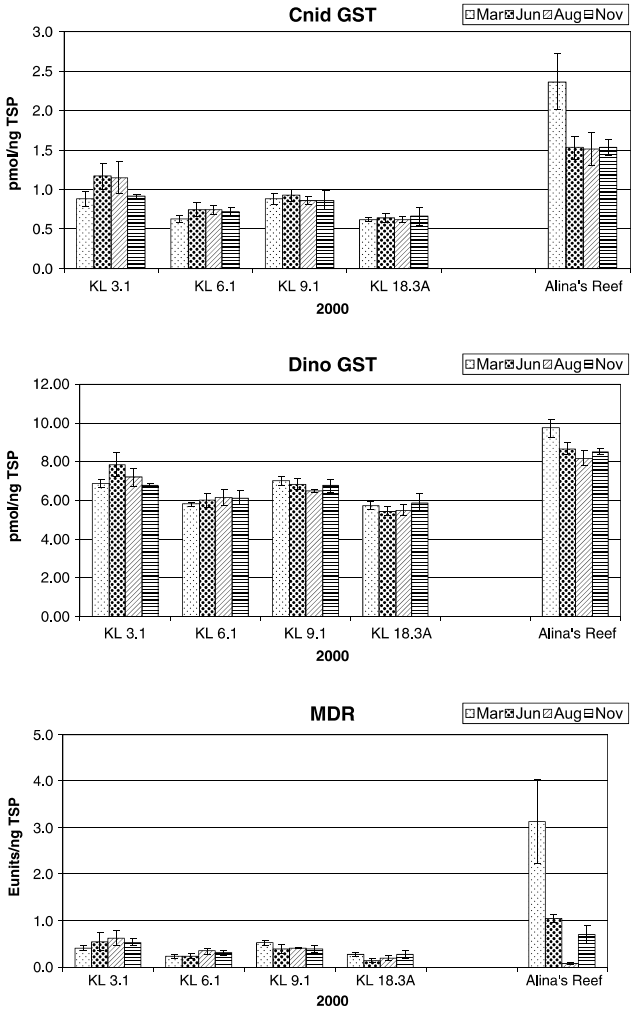

Fig. 7. Xenobiotic Response. Levels of cnidarian (Cnid) and dinoflagellate (Dino) biomarkers associated with Xenobiotic Response from all five coral reef sites that were sampled quarterly. Bars show untransformed means (±SE), N = five corals per site/date. Time point of sampling at each site from left to right: March, June, August, and November.

may be a chemical xenobiotic. To test this hypothesis, we examined three cellular parameters that would be significantly modulated by a xenobiotic: cnidarian GST, dinoflagellate GST, and the P-glycoprotein 180 homologue (multixenobiotic resistance protein, MXR-MDR), from both species. Cnidarian GST levels were significantly elevated in March at Alina's Reef (Welch ANOVA, $p < 0.001$), and continued to be significantly elevated (Welch ANOVA, $p < 0.01$), Fig. 7. Dinoflagellate GST was elevated at Alina's Reef in March, as well as the rest of the year, but this elevation was not as pronounced as the cnidarian GST (Welch ANOVA, $p < 0.001$), Fig. 7. Multixenobiotic resistance protein, MXR-MDR, is an ABC cassette protein that lowers the intracellular concentration of toxic compounds below their level of toxicity (Bard, 2000). Increase of this protein is usually only in response to an organic xenobiotic (Bard, 2000; Sauna et al., 2001). Unfortunately, this antibody binds both the cnidarian and dinoflagellate isoforms, hence results are a composite of MDR expression in both. MDR levels were significantly elevated in March at Alina's Reef above all other sites at all time

points (Welch ANOVA, $p < 0.001$), Fig. 7. MDR levels at Alina's Reef decreased in June and were not significantly different than MDR levels Sites 1–4 throughout the rest of the year.

4. Discussion

Levels of almost all of the cellular diagnostic markers for corals sampled at Sites 1–4 near Molasses Reef were not significantly different from one another during any of the sampling time points (ANOVA followed by Tukey–Kramer HSD test, $p > 0.05$). The significant difference in coral biomarker levels between Alina's Reef and Sites 1–4, coupled with field observations, suggests that the coral decline observed in the Upper Keys may be related to local factors rather than large climatic actions (e.g. El Nino). Though we are able to ascertain that the localized stressor induces an oxidative stress, is disruptive to mitochondrial 'nominal' equilibrium, and elicits a strong xenobiotic detoxification response, the exact identification of the stressor remains unknown. The inability to more accurately identify the stressor could be alleviated by increasing the scope of the cellular biomarkers in corals, and by contaminant/chemistry analysis. For example, assays for cnidarian metallothioneins, cnidarian methyl arsenic reductases, dinoflagellate sulfate permease, dinoflagellate nitrate reductase, and dinoflagellate calcium-dependent alkaline phosphatase could be developed to aid in deducing the identity profile of the stressor(s). Alternatively, assays for chemical compound adducts with DNA or protein could be developed to not only identify the agent, but also establish that it is having a direct biological effect (Downs et al., 2002b). Environmental chemistry analysis, along with contaminant analysis (e.g., endosulfan, atrazine, mercury, etc.), would tremendously increase the power in determining stressor identity from among a number of potential suspects. Laboratory toxicological studies (e.g. Downs et al., 2000; Downs, 2005; Hawkridge et al., 2005) may provide further corroborating evidence as to the identity of the putative stressor, especially since the coral samples have been archived and can be analyzed with new assays as they are developed.

5. Conclusions

We have provided evidence that coral decline in the Florida Keys may, in part, be rooted with local factors and, at least for Alina's Reef, be associated with xenobiotic exposure. Further development of cellular diagnostics for coral health assessment and their integration with other disciplines represents a powerful tool for (a) providing resource managers valuable information as to the health condition of specific coral reefs and (b) providing evidence concerning the identity of putative stressors, allowing the development of defensable mitigation strategies (Downs et al., 2005). The approach also potentially allows the fate of coral reefs to be forecasted in light of stress events. In this regard, good prognostic markers for morbidity/mortality are those that are usually directly associated with cellular damage. Protein carbonyl, lipid peroxidation products, and DNA damage products have been demonstrated as good prognostic indicators for corals, while stress defense proteins and solutes have demonstrated value as indicators of stress susceptibility (Downs et al., 2002a; Fauth et al., 2003). Further development of cellular biomarkers in corals and other coral reef species could greatly enhance the ability of resource managers to make ecological forecasts.

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