

Agenda

Environmental Marine Biotechnology: Constituent's Workshop

National Sea Grant & South Carolina Sea Grant

Environmental Marine Biotechnology Program: 'Assessing the Health of Coral Reef Ecosystems in the Florida Keys Using an Integrated Molecular Biomarker system'

Saturday January 17, 2004

9:00 am	<i>Continental Breakfast</i>	<i>Casa Blanca I</i>
9:30 am	Welcome Overview of the Project and Findings	<i>Cheryl Woodley</i>
9:50 am	Cellular Diagnostics (CDS) – Theory and Practice	<i>Craig Downs</i>
10:10 am	Study Site Assessment – Ecological Perspective	<i>Beth Fisher</i>
10:25 am	White Grunts (<i>Haemulon plumieri</i>) – CDS	<i>John Fauth/Craig Downs</i>
11:00 am	<i>Break</i>	
11:15 am	Bicolor Damselfish (<i>Stegastes partitus</i>) – CDS	<i>John Fauth/Craig Downs</i>
12:00 noon	Snails (<i>Coralliophora abbreviate</i>) –CDS	<i>John Fauth/Craig Downs</i>
12:45 pm	<i>Lunch - Buffet at Hotel</i>	
2:00 pm	Coral (<i>Montastraea annularis</i>)- CDS	<i>John Fauth/Craig Downs</i>
2:45 pm	Organism Response: Lesion Healing and Forams	<i>Beth Fisher</i>
3:15 pm	<i>Afternoon Break</i>	
3:30 pm	"Reef Risk Assessment: Satellites to Molecules"	<i>Pam Hallock Muller</i>
4:00 pm	Charge to Working Groups "Merging Science and Technology for Management"	<i>Malcolm Meaburn</i> <i>Cheryl Woodley</i>
4:15 pm	Working Groups Meet	
6:00 pm	Adjourn	
7:00 pm	<i>Dinner at The Pilot House Restaurant</i>	
9- 11pm	Meeting Room Open for Impromptu Meetings	

Agenda (con't)

Sunday, January 18

8:30 am *Breakfast*

9:00 am Working Groups Re-convene

10:30 am *Break*

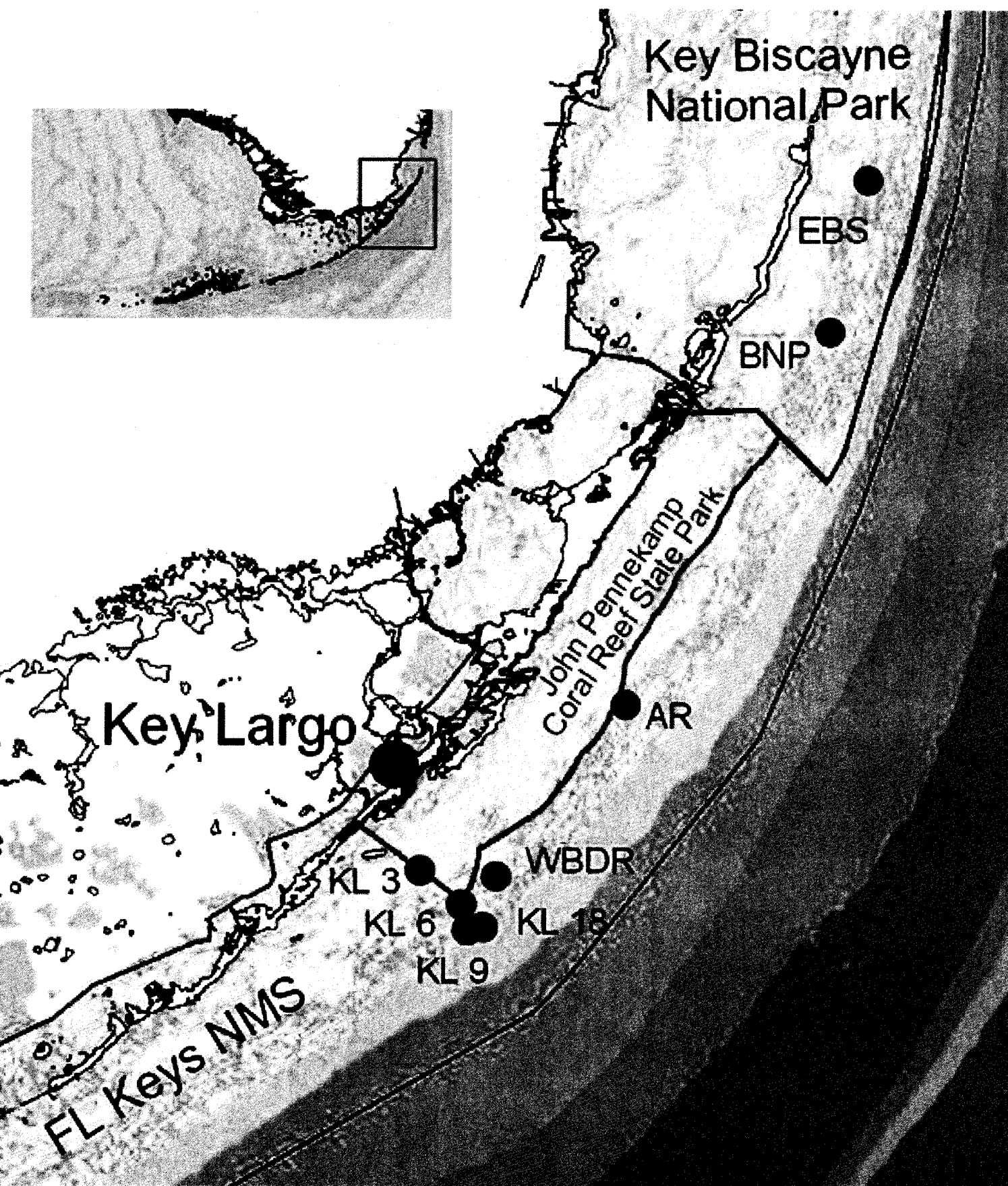
10:45 am Working Groups

11:15 am Group Leader Reports

11:45 am Wrap up and Observations

*Malcolm Meaburn
Cheryl Woodley*

12:00 noon Adjourn



South Carolina Sea Grant Consortium

Project Report

Annual Progress Report

Reporting Period: From: October 8, 2000
To: January 31, 2002

Project Number: R/MB-2

Date of Report: March 28, 2002

Project Title: **Environmental Marine Biotechnology - Assessing the Health of Coral Reef Ecosystems in the Florida Keys using an Integrated Molecular Biomarker System**

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Collaborators:	Richard Curry, Science Coordinator, Biscayne National Park, Homestead, FL 33033 John Halas, Florida Keys National Marine Sanctuary, Key Largo, FL 33037 David Makepeace, Dept Biology, Coral Shores High School, Tavernier, FL	

Objectives: We will test an integrated Molecular Biomarker System (MBS) designed to diagnose whether an organism is stressed, and identify the likely stressor(s) (e.g., heat stress, pesticides, pathogens). Our goals are to:

- 1) use the MBS to characterize the health of a coral reef ecosystem in the Florida Keys;
- 2) verify that the MBS can detect and characterize subtle and chronic effects of environmental stressors on this ecosystem;
- 3) determine if point-source pollutants or global climate changes (e.g., increased ocean temperatures or UV-B radiation) are stressing coral reef ecosystems;
- 4) compare the precision, sensitivity and prognostic capabilities of the MBS to those of traditional measures of ecosystem health, and
- 5) encourage the participation and understanding of the general public and scientific, industrial, and managerial communities in using marine biotechnologies to assess and manage the health of coral reef ecosystems.

Project Results (Results to Date, if Annual Report): Please summarize results of your research or education project. Please structure your response by Project Objective. (Cite non-economic impacts or results, quantifiable or anecdotal.) Be complete. Attach additional pages if necessary. Also, please note any unanticipated problems in meeting project objectives.

SUMMARY RESULTS TO DATE: Our interim results indicate that this technology can be used to characterize coral health in defined areas of the Florida Keys (Obj. 1); distinguish between global-level stressors (e.g., El Nino/La Nina effects) and local-level stressors (e.g., agricultural runoff), (Obj. 2 & 3); and help predict the condition of corals several months before more obvious symptoms appear (e.g., coral bleaching or coral death) (Obj 3 & 4). Additionally, comparisons of coral lesion healing with biomarker response shows significant correlations between levels of biomarker response (representative of the cellular physiological status) and measures traditionally used to assess ecosystem health (Obj. 4). These results support our hypothesis that a coral, which the MBS indicates to be more stressed, will be less likely to regenerate than a coral, which MBS indicates to be less stressed. These preliminary findings have been presented to user groups that include both state and federal resource managers, coral biologists and representatives from the general public at a workshop in March 2002. Participants provided valuable input that included increasing the spatial scale of the project in year two and conducting laboratory challenge experiments of possible stressors (Obj. 5).

Objective 1: Use the MBS to characterize the health of a coral reef ecosystem in the Florida Keys.

Objective 2: Verify that the MBS can detect and characterize subtle and chronic effects of environmental stressors on this ecosystem.

Objective 3: Determine if point-source pollutants or global climate changes (e.g., increased ocean temperatures or UV-B radiation) are stressing coral reef ecosystems.

To address objectives 1-3 we have compared levels of MBS parameters from 2001 collections with previous data collected in 1999 and 2000 in the same locations.

Recently, the Molecular Biomarker System has been renamed by EnVirtue Biotechnologies, Inc. due to improvements and additions to the original MBS. The improved system is now referred to as the ECDS (EnVirtue Cellular Diagnostic System, *ECDS*-patent pending). Both the original MBS and now the ECDS were developed to focus a comprehensive array of biotechnologies on the diagnosis of a variety of ecosystem health issues. However it was specifically tailored to coral physiological health and discerning the causes of coral reef system declines (Downs et al., 2000; Woodley et al., 2001). The ECDS specifically is used to measure changes in cellular parameters and thus allows assessment of cellular-physiological condition, monitoring of cellular stress responses, identification of putative stressors, and forecasting outcomes of environmental problems. The specific cellular and molecular parameters used to assess physiological condition, include (but are not limited to) membrane integrity and composition (e.g., lipid peroxidation products), anti-oxidant redox potential (e.g., glutathione redox status), molecular chaperone activity (e.g., heat-shock proteins 60 & 70), enzymatic anti-oxidants (e.g., catalase, superoxide dismutases, glutathione peroxidases), stress-signaling pathways (e.g., MAPK, JANK), xenobiotic detoxification pathways (e.g., cytochrome P450 family, P-glycoprotein 160), metal-regulatory proteins (e.g., metallothionein, ferritin, porphyrin), protein status and turnover (e.g., ubiquitin, protein carbonyl formation), and genomic and

translational integrity (e.g., DNA abasic phosphate site formations). These parameters quantify specific cellular physiological functions, including (1) whether the structural integrity of the cell is compromised, (2) the type or nature of the stress (e.g., oxidative stress, metal stress, salinity stress), and (3) whether defenses have been mounted against a particular stress (i.e., pesticide, acidity, heavy metal, PAH).

In this study, we specifically applied the ECDS to corals in the Florida Keys and demonstrated that the ECDS could distinguish whether a local coral population of *Montastraea annularis* was being stressed by a global stressor (e.g., high sea-surface temperatures; Fig. 1) or by a stressor that is local in nature (Fig. 2). In conjunction with other technologies and monitoring methods, this biotechnology was able to identify potential stressor(s) responsible for the decline (Fig. 3). The ECDS also possesses the ability to predict the progression of a health condition based on key diagnostic markers (Fig. 4).

In Figure 1, *M. annularis* scleractinian Hsp60 and ubiquitin data from the 1999 sampling project can be diagnostically interpreted that corals at all four depths were experiencing a protein denaturing stress, as indicated by increased ubiquitin levels (a key component of a pathway for degrading 80% of the proteins in the cell), that is positively correlated with abnormally high sea surface temperatures that peak in the months of July and August (Downs et al., in review A). Hsp60 (for description of function, see Downs et al., 2000) data in 1999 corroborate this diagnostic interpretation. Though the extent of cellular damage differed significantly with depth, the data support the argument that coral cellular damage at all four sites was the result of a global stressor (La Nina sea-surface temperature

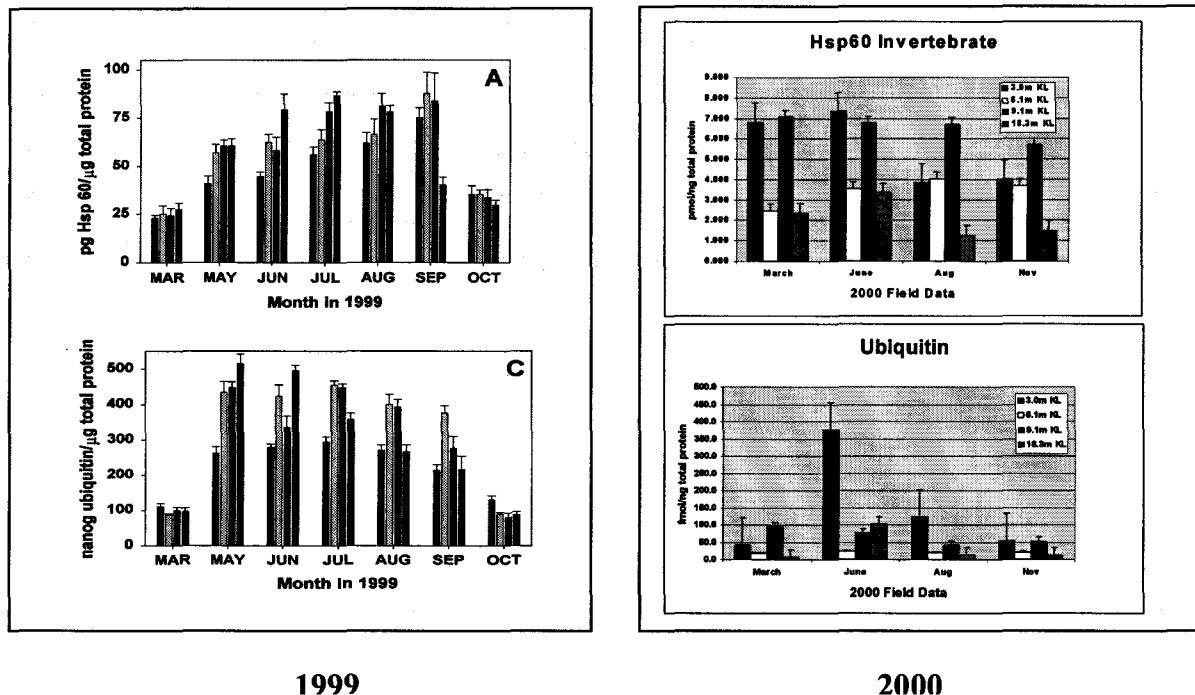


Figure 1. The same coral colonies from four depth sites were sampled on a monthly basis in 1999 and a quarterly basis in 2000. Hsp60 reflect chaperonin levels in the scleractinian; mean concentrations varied significantly with depth, month, and the depth x month interaction in 1999 (repeated measures MANOVAs: all $F > 2.56$, $P < 0.02$). Ubiquitin levels reflect the rate of protein degradation, which varied significantly with depth, month, and the depth x month interaction (repeated measures MANOVA: all $F > 8.80$, $P < 0.0001$). Bars show untransformed mean (± 1 SE) biomarker concentrations at each depth: for 1999 panel, black = 3.0 m, grey = 6.1 m, red = 9.1 m, blue = 18.3 m. Sites are from a four-mile long transect off the eastern shore of Key Largo.

effects). In 2000, the patterns of both parameters are radically different than that of observed for 1999 and are not correlated with sea-surface temperatures (Woodley et al., in preparation). In March 2000, corals at 3 meters were not experiencing a protein denaturing cellular condition, but were experiencing non-adverse changes in mitochondrial function. In June 2000, corals at the 3m site indicated that they were experiencing a cellular stress that was adversely affecting mitochondrial function. The diagnostic interpretations for both 1999 and 2000 are corroborated by other diagnostic biomarker data. In summary, the cellular stress experienced by corals at all four sites was the result of global stressor while cellular damage experienced by corals at the 3.1m and 9.1m sites support the argument that a local stressor is impacting these two sites (and that the stressor is different for these two sites – Woodley et al., in preparation). In 2000, using only three diagnostic markers (out of 24 biomarkers assayed for each coral sample), we determined that a coral reef site in Biscayne National Park (BNP) was experiencing a severe cellular stress and that it was most likely generated by an electrophillically-modifiable xenobiotic (e.g., a fungicide, an organometalloid, endosulfan) (Fig. 2 & 3). The extremely high-level of ubiquitin indicates severe rates of protein turnover; this interpretation was corroborated by five other cellular biomarkers. The level of ubiquitin in March 2000 at the BNP site has been suggested to be near the maximal threshold capacity for this coral species – massive cellular deterioration is beginning to occur and coral death could be predicted (Downs and Woodley, in preparation). In August 2000, significant, punctuated coral coverage loss at

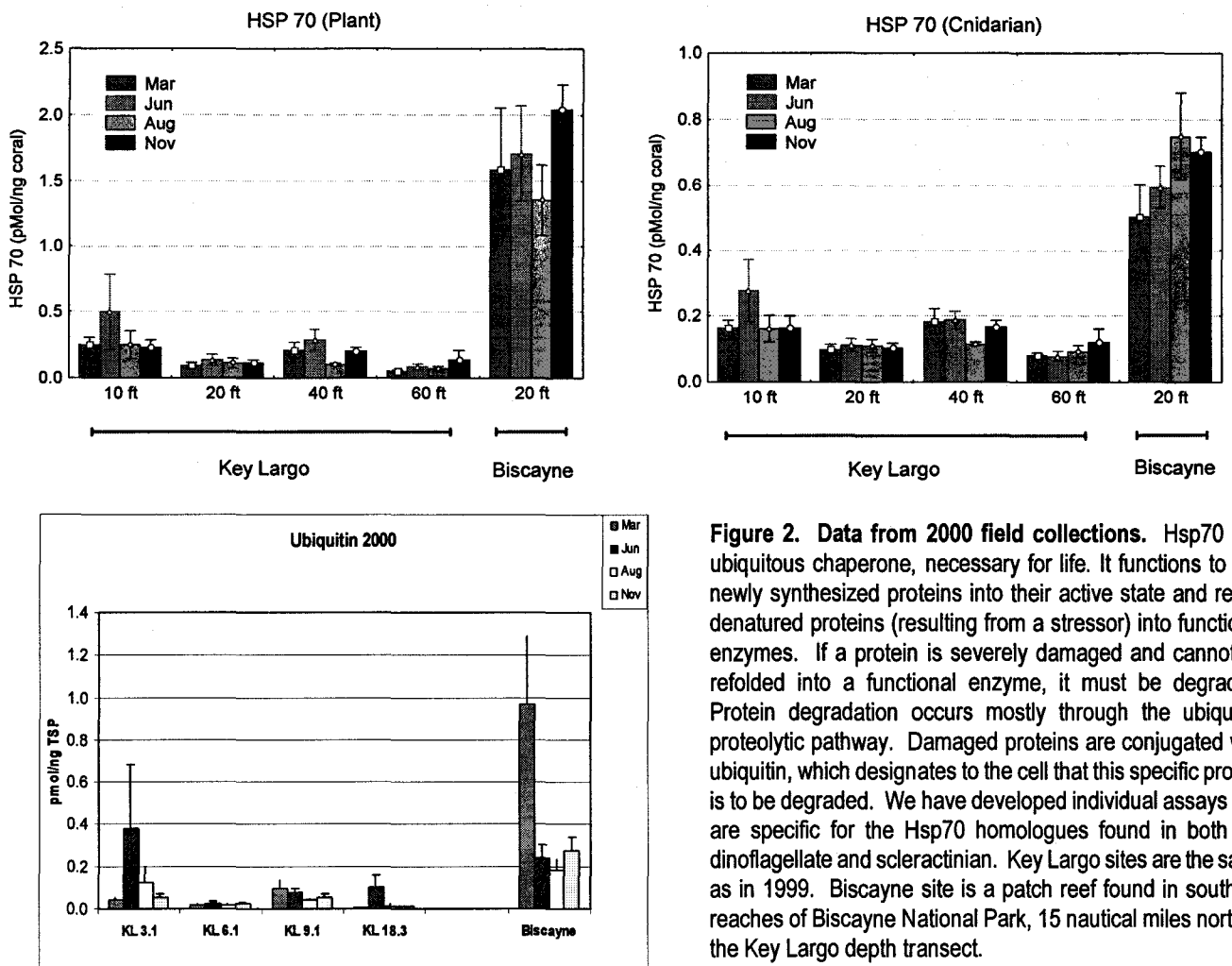


Figure 2. Data from 2000 field collections. Hsp70 is a ubiquitous chaperone, necessary for life. It functions to fold newly synthesized proteins into their active state and refold denatured proteins (resulting from a stressor) into functional enzymes. If a protein is severely damaged and cannot be refolded into a functional enzyme, it must be degraded. Protein degradation occurs mostly through the ubiquitin-proteolytic pathway. Damaged proteins are conjugated with ubiquitin, which designates to the cell that this specific protein is to be degraded. We have developed individual assays that are specific for the Hsp70 homologues found in both the dinoflagellate and scleractinian. Key Largo sites are the same as in 1999. Biscayne site is a patch reef found in southern reaches of Biscayne National Park, 15 nautical miles north of the Key Largo depth transect.

the BNP site was observed – no observable coral coverage degradation was observed in March 2000. This partially unidentified stressor adversely affected both the scleractinian cellular physiology and the dinoflagellate cellular physiology (Fig. 2). Data presented in Fig. 3 can be interpreted that corals at the BNP site were responding to a xenobiotic stressor and that the response pathway included a monooxygenase catalytic reaction at the site of olefinic double bonds of the xenobiotic, the conjugation of glutathione to the xenobiotic by glutathione-s-transferase, and cellular exclusion of the GSH-conjugated xenobiotic by a P-glycoprotein 140/160 pump action (a.k.a. MDR: multi-drug resistance gene) (Woodley et al., in preparation; Downs et al., in preparation B; not all data shown for this interpretation).

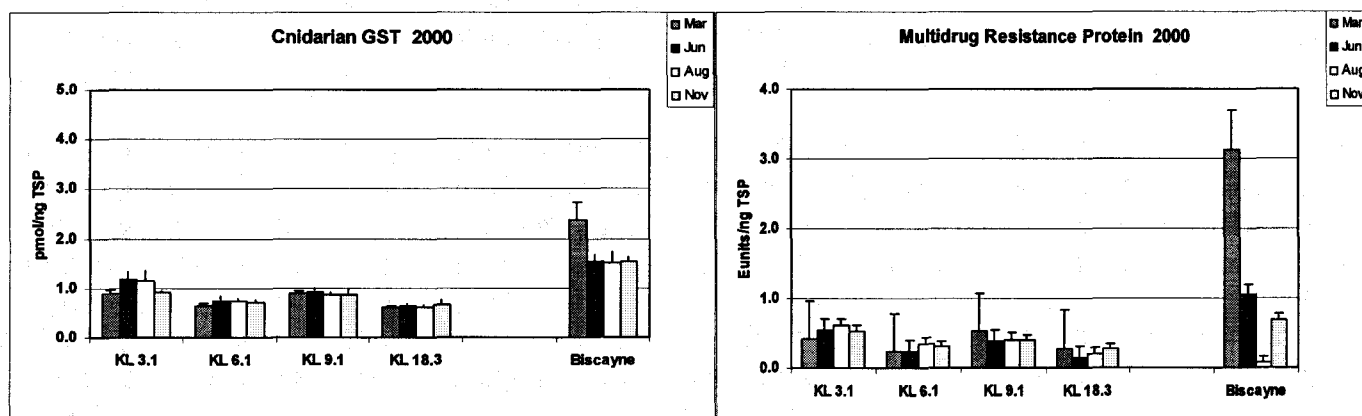
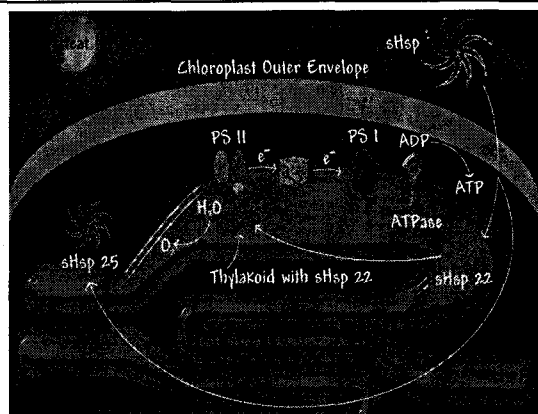
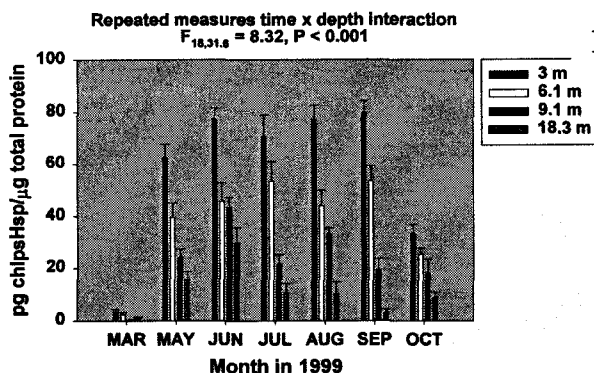


Figure 3. Data from 2000 Field collections. GST Invertebrate = scleractinian homologues of glutathione-S-transferase. GST is an enzyme that will conjugate a xenobiotic with reduced glutathione so that the xenobiotic can easily managed by the cell. MDR = P-glycoprotein 160, a member of the ABC family of proteins that is up-regulated when an organism has been exposed to specific classes of xenobiotics. Its function is to detoxify the cell of xenobiotics by pumping these xenobiotics out of the organism. Site locations are the same as described in Figure 2.

A



B



C

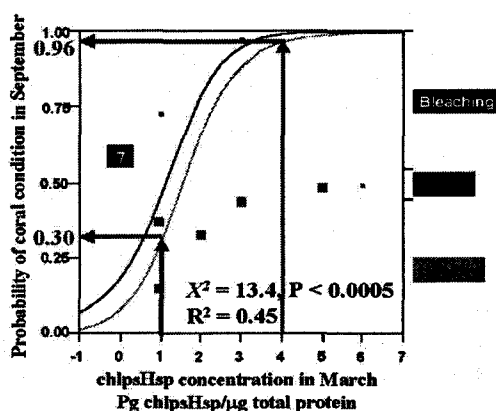
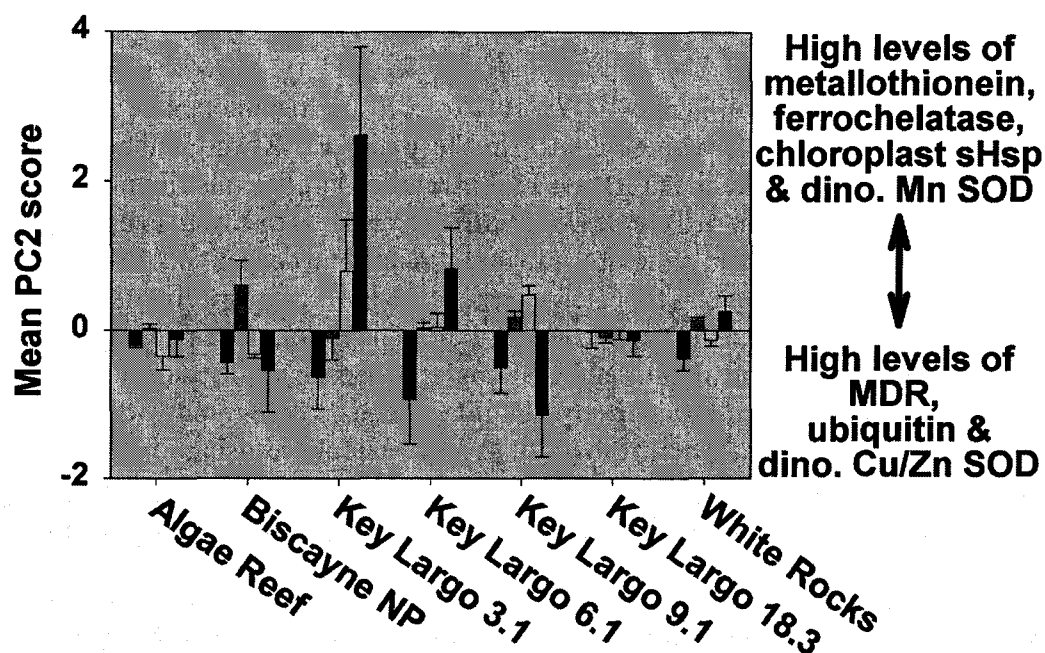


Fig. 4 Panel A – Function of the chloroplast small heat-shock protein (ChlpsHsp). This protein is only induced when photosystem II is being damaged. It is a major adaptation of photosynthesis against heat stress, oxidative stress, and photoinhibition (Downs et al., 1999a, b) Panel B – Levels of ChlpsHsp in dinoflagellate of *M. annularis*. Coral samples and sampling scheme the same as in Fig. 2. (Downs et al., in rev.) Panel C- Logistic regression analysis of probability of the level of chlpsHsp in March to predict coral bleaching in September when sea surface temperatures reached 31°C in August (Fauth et al., in prep.)

In 2001, we continued to detect responses that indicated local stressors. Using a principal component analysis the following patterns emerged: The Key Largo 3.1 m site differs greatly from all the other six sites. The huge upward spike in October suggests a response to metals dominated over a xenobiotic response. The Key Largo 6.1 m site has a similar profile, only not as pronounced. The one explanation is sedimentation and runoff contributed to these responses. Although we cannot unambiguously identify the specific stressor at this time we do know that it dramatically affects the algal component of the coral, as indicated by markers specific for the chloroplasts and algal mitochondria and is likely associated with runoff and rainfall events. The effect of sedimentation will be tested using sedimentation data collected from sediment traps. In addition, except for the most offshore site, all other sites had negative PC2 scores in March, suggesting responses to a xenobiotic predominated over a metal stress. Again one possible explanation is pesticide runoff. This will be further investigated by examining whether correlations exist between molecular biomarker parameter levels and water chemistry parameters (chl a and/or pigments).

2001



Objective 4: Compare the precision, sensitivity and prognostic capabilities of the MBS to those of traditional measures of ecosystem health.

The condition of the corals at selected sites in Biscayne National Park (BNP) and in the upper Florida Keys National Marine Sanctuary (FKNMS) is being assessed at multiple scales in order to compare the precision, sensitivity and prognostic capabilities of the MBS with measures traditionally used to assess ecosystem health. Community-scale condition of selected patch reefs was assessed using the well-established Atlantic and Gulf Rapid Reef Assessment (AGRRA, Ginsburg et al., 2000). This protocol determines the condition of reefs by evaluating major benthic taxa that comprise them: coral and algae. The condition (i.e., mottling or bleaching) of populations of a key symbiont-bearing foraminifer (*Amphistegina gibbosa*), living in the vicinity of the corals, is also being monitored according to Hallock et al., 1995. These data will be used to determine if there is a correlation between bleaching stress in the foraminifer and bleaching or other stress responses in corals. This information will help determine if foraminifer can be used as a surrogate for study of mechanisms of coral bleaching. Individual-scale studies, include monitoring lesions on the coral (Meesters et al., 1997) and the assessment of the overall condition (i.e., bleaching, disease, overgrowth, etc.) of the sampled corals. These assessments are compared to measures of health status taken at the cellular physiological level in coral (*Montastraea annularis*), fish (*Haemulon plumieri* and *Stegastes partitus*), algae (*Halimeda opuntia*), and snails (*Coralliophora abbreviata*) using a Molecular Biomarker System (MBS) (Downs et al., 2000; 2001). The Molecular Biomarker System assesses indicators of cell integrity indicative of stressed or non-stressed conditions. Environmental data, including continuous water temperature measurements (using HOBO data loggers) and nutrient levels (taken at the time of biological sampling), sediment-trap data, and data from other ongoing monitoring studies, are also being collected. The environmental data will be

analyzed in conjunction with community, population, coral condition and molecular data to develop a more comprehensive overview of coral ecosystem health and provide evidence for the underlying stresses.

To date, we have compared coral lesion healing with levels of cellular parameters at one site in Biscayne National Park (Alina's Reef) with five sites in the upper FL Keys National Marine Sanctuary that represent both a depth gradient (3.1m – 18.3 m) and geographic distribution (3 sites each at 6.1m). To accomplish this we tagged corals (*Montastraea* complex) that were to be sampled. Corals were sampled using a 1.5cm punch, removing an approximate 3mm deep divot of tissue from the colony surface. The sampling employed a repeated measures design on a quarterly basis in 2001 (March/April, June, August/September and October/November). The lesions (defined as an area on the colony with no live coral tissue) were monitored by photographing each lesion using a Nikonis V camera with a close-up adapter at each of the quarterly sampling events. Photographs were scanned to digital images, then the area and perimeter of the lesions were calculated using image analysis software. Tissue samples were analyzed by ELISA for 20 cellular parameters included in the MBS. Our hypothesis was that a coral, which the MBS indicates to be more stressed, will be less likely to regenerate than a coral, which MBS indicates to be less stressed.

The March sampling lesions from the Key Largo 3 m site experienced a large degree of regeneration with some lesions closing completely (Fig. 5A and 6A), whereas other sites, such as the Key Largo 10 m site have experienced very little regeneration with some lesions showing increases in mortality (Fig. 5B and 6A). However, the June sampling lesions from the Key Largo 10 m site were able to regenerate the best relative to the lesions from the shallower corals at the Key Largo 6 m and 3m sites (Fig. 6B). Algae Reef (site 6) and White Banks (site 5) showed the greatest amount of regeneration year round relative to the other two 6 m sites, Alina's Reef and the Key Largo 6 m site, which show very little change year round (Fig. 7A and 7B).

Results of a backward stepwise regression, to determine which of the cellular parameters explained significant variation in coral re-growth indicated that re-growth was correlated with depth plus five of the cellular parameters, MDR (multidrug resistance protein), dinoflagellate heat shock protein 60, cnidarian Cu/Zn superoxide dismutase, dinoflagellate Mn superoxide dismutase and dinoflagellate glutathione peroxidase. Corals with high levels of plant Hsp 60 and plant glutathione peroxidase healed more quickly, both indicators of a healthy status. While lesions in corals with high MDR, cnidarian CuZn superoxide dismutase, and plant Mn superoxide dismutase levels healed more slowly, suggesting they were stressed with a xenobiotic, thus allocating less of their energy to regeneration. These analyses indicate that corals located at Algae Reef showed significantly higher re-growth of lesions than at the 9.1m and 18.3m sites off of Key Largo.

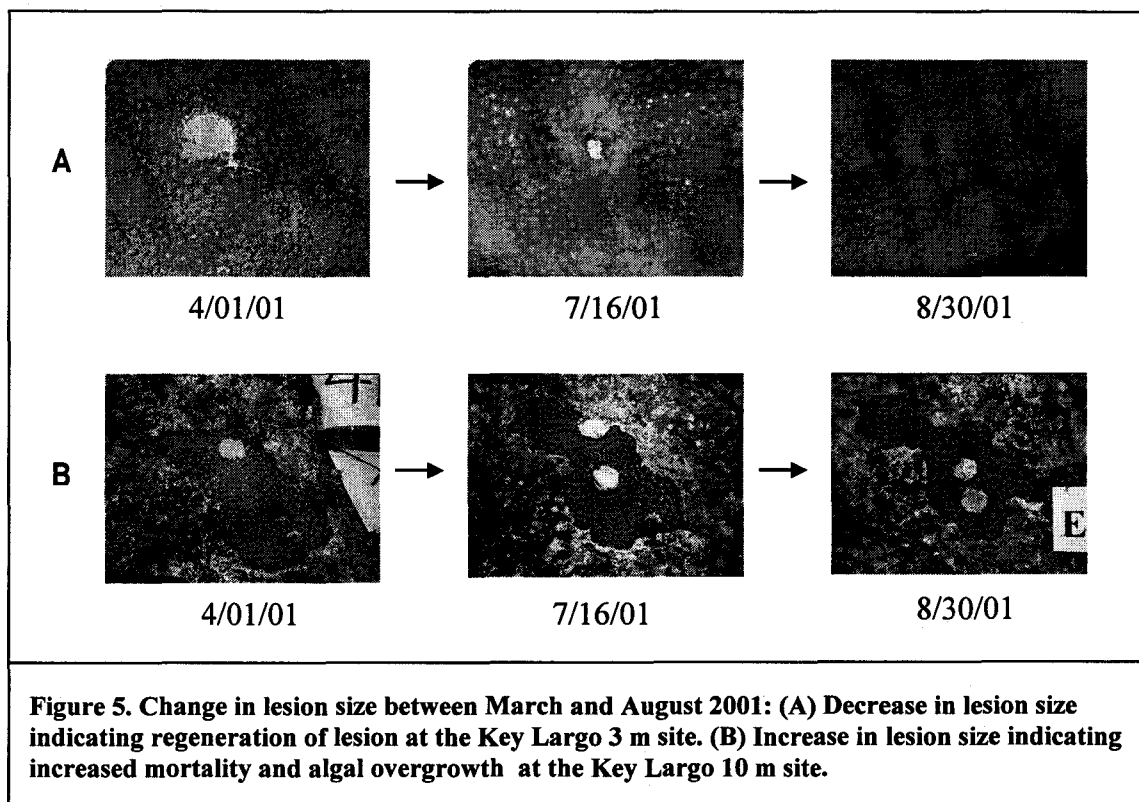


Figure 5. Change in lesion size between March and August 2001: (A) Decrease in lesion size indicating regeneration of lesion at the Key Largo 3 m site. (B) Increase in lesion size indicating increased mortality and algal overgrowth at the Key Largo 10 m site.

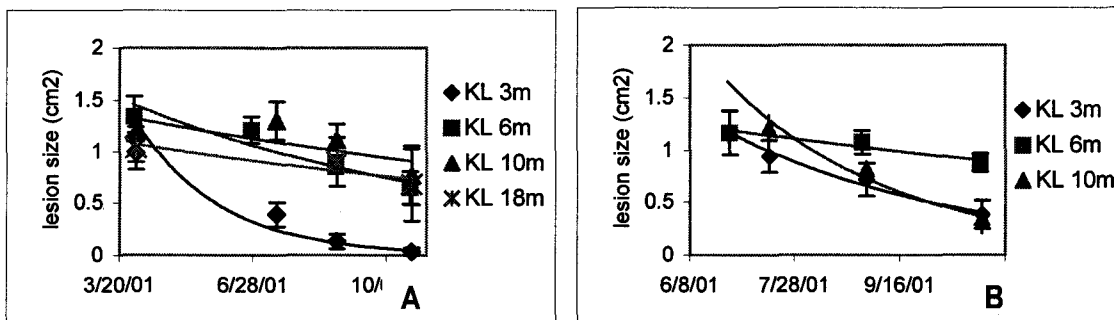


Figure 6. Change in mean lesion size (cm² ± SE) along a depth gradient of 3m, 6m, 10m, and 18m in Key Largo. Lines fit to an exponential model. (A) March sampling lesion (B) June sampling lesion

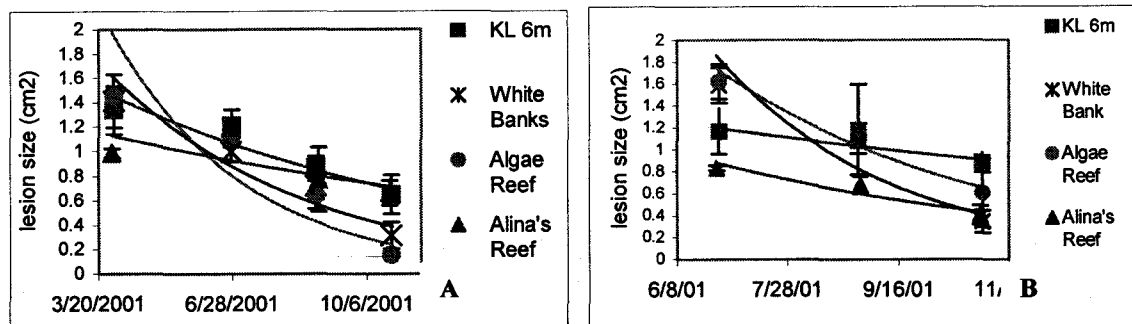


Figure 7. Change in mean lesion size (cm² ± SE) for the four 6 m sites including Key Largo 6m (site 2), White Banks (site 5), Algae Reef (site 6), and Alina's Reef (site 7). Lines fitted to an exponential model. (A) March sampling lesion (B) June sampling lesion

Objective 5: Encourage the participation and understanding of the general public and scientific, industrial, and managerial communities in using marine biotechnologies to assess and manage the health of coral reef ecosystems.

Throughout this project we have had participation from individuals from many walks of life. We have had divers participate on various missions that included individuals from a local high school marine biology class, retirees from the local community, resource managers, graduate student volunteers and industry. Through these interactions we have been able to communicate and educate the participants about the novel technology we are testing, the similarity to what is used in modern biomedicine and the prospects this technology brings to understanding coral reef degradation and developing scientific based strategies to combat them. We have also engaged the scientific and resource management community in evaluating and critiquing our data and experimental design in a recent workshop (March 15, 2002). We had representatives from academia, the State of Florida, Biscayne National Park, USGS, FL Keys National Marine Sanctuary, National Undersea Research Program, industry, Mote Marine Laboratory and the National Ocean Service. They reviewed our data and provided critical input to our second year design.

References:

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Cellular Diagnostics and Its Application to Aquatic and Marine Toxicology

Craig A. Downs

Theory

Biomarkers indicate the status or condition of a specific biological property. In principle, biomarkers are generally classified into three categories: biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility^{1,2,3}. Biomarkers of exposure indicate that the organism is or has been exposed to a specific class of xenobiotics or environmental conditions. Biomarkers of effect are indicative of alterations in molecular and cellular processes of an organism as a result of a xenobiotic or environmental exposure. Biomarkers of susceptibility reflect the risk for which an organism may acquire an altered physiological condition. The major difficulty in the application of biomarkers within toxicology is defining and linking different biomarkers within and between categories into an integrated system that can (1) identify a stressor or stressors affecting an organism, (2) distinguish between different physiological conditions (3) elucidate the mechanism of toxicity as a result of the interaction, and (4) predict the fate of the interaction².

Cellular Diagnostics is a systematic approach to defining and integrating biomarkers of exposure, effect, and susceptibility based on their functionality within a cell and how alterations in the behavior of a single or set of cellular parameters (biomarkers) may reflect overall cellular operation or performance. The cell is a dynamic system comprised of both macro- and micro-structures and processes. Many of these sub-cellular processes are key metabolic pathways and cellular structural components that are essential for maintaining cellular operations and homeostasis. These cellular metabolic pathways and structural operations can be divided into categories or sub-systems of cellular integrity and function which can be further defined by discrete parameters (Table 1). The behavior of these components and processes defines the physiological condition of the cell. Hence, changes in the behavior of the cellular components/processes are, in effect, changes in the physiological condition of the cell.

Adapting the concept of Cellular Diagnostics to aquatic toxicological assessments first requires defining cellular toxicity. The Cellular Diagnostic approach posits that changes in *cellular integrity* and *homeostatic responses* are fundamental end-points in discriminating whether a xenobiotic or environmental factor is affecting the cell. The terms *cellular integrity* and *homeostatic responses* can be partially and stipulatively defined by the some of the categories in Table 1. It should be noted that these *definiens* of *cellular integrity* and *homeostatic responses* are used because they are measurable by the present technology and are well understood. As our understanding and technology of cell biology evolves, so too will the categories and parameters that define *cellular integrity* and *homeostatic response*.

Each category is defined by a functional process and the physical components that are essential to that process. For example, protein metabolic condition can be defined, in part, by many of the RNA, protein and enzymatic components (sub-systems) that play a role in protein synthesis, protein maturation, and protein degradation. To use the category of protein metabolic condition as an example, the three sub-systems of protein metabolic condition can be defined and measured by the following parameters:

Protein Synthesis

- aminoacyl-tRNA synthase
- eIF1 (translation initiation factor 1; stress inducible A121/SUI1)
- p67 (modulator of eIF2 activation)

Protein Chaperoning

- Hsp72 B' (inducible Hsp70 cytosolic homologue)
- Grp75 (inducible Hsp70 mitochondrial homologue)
- Grp78 (Hsp70 endoplasmic reticulum homologue)
- Hsp60
- Hsp90 α (chaperonin)

Protein Degradation

- Ubiquitin (free and conjugated)
- Ubiquitin activase (E1)
- Ubiquitin ligase (E2)
- Lon (mitochondrial-localized protease)

These molecular entities were selected as Cellular Diagnostic parameters for protein metabolic condition because changes in the concentration or activity of these entities may reflect a change in that sub-system. The other categories that define cellular integrity and homeostatic response are similarly structured (Table 1).

Shifts in the steady-state rates or levels of these parameters are indicative of a shift in the equilibrium of these sub-systems. These shifts contribute to defining the cellular condition. Deviation of the behavior of a specific parameter from the reference is an altered state. An altered state is defined as a pathology or 'diseased condition' for the individual (or population) only if that phenotype is associated with condition that adversely affects *performance*⁴. *Performance* is defined by reproductive fitness, metabolic condition, immuno-competence, genomic integrity, and other similar aspects (Table 1). Hence, a pathology or diseased condition may be defined by an abnormal vital function involving any structure, process, or system, whether it is for a cell, individual, or population. In the context of toxicology and Cellular Diagnostics, a diseased state is any condition where there is deterioration in cellular integrity and a reduction in homeostatic capacity.

Cellular Diagnostics is analogous in its methodology to clinical and engineering diagnostics, both in theory and practice. Three elements or 'concepts' are inherent to the process of diagnosis which include (1) mastery of the literature (scholarship) and mastery of technique, (2) observation (data) and history of the subject, and (3) the reasoning and interpretative process⁵. The criteria established in all three elements of the diagnostic process must first be met in order to achieve the goals of Cellular Diagnostics: to detect changes in the subject and to relate or designate an understanding of what those changes mean in terms of the quality of cellular 'health'.

The first element of the diagnostic process requires a thorough knowledge of the principles and facts of biochemistry, cell physiology, and physiology, and includes mastering the literature pertaining to each of the cellular parameters that are to be examined⁶. For example, "Hsp70" (heat-shock protein 70) is not just "Hsp70"; there are constitutive and inducible isoforms and isoforms that have distinctive sub-cellular localizations that possess slightly different functional properties⁷. Knowing the functional role of each isoform is necessary for not only deriving an accurate interpretation/diagnosis of what the data means, but to also design the appropriate tests and assays in a test array. The other aspect of this element is achieving a level of skill and competence in performing the assay protocols and in handling of the specimen. Additionally, it requires the ability to modify or optimize these standardized protocols to account for the nuances inherent to the sample matrix. For example, to successfully (*i.e.*, an acceptable level of confidence in the data produced) conduct a SDS-PAGE/western blot immuno-assay for protein carbonyl residues on blood serum from sea otters and on polyp tissue of *Porites lobata* (a species of hard coral), different ingredients in the buffers as well as different PAGE running conditions are required for each species. An understanding of the matrix composition of the sample is crucial for optimizing assay protocols to provide an acceptable level of confidence in the accuracy of the data produced.

The second element of the diagnostic process is composed of two types of observations: *anamnesis* and test evaluation. *Anamnesis* or 'history' is the process of recognizing and understanding the context in which the samples will be analyzed. *Anamnesis* is the act of taking into account a patient's history. In the context of Cellular Diagnostics and toxicology, it's the appreciation of an organism's life history, the organism's current state of development, the motives underlying the investigation, and the justification for sampling and testing specific tissue type(s) or the whole organism. Cellular Diagnostics is based on the premise that there is a hierarchical relationship between cellular level behavior and phenomena that occurs at the tissue, organismal, and population level⁸. The toxicological investigation may be focusing on the question as to why an exposure to a particular xenobiotic decreases the fecundity of that organism or it may be focused on why mortality occurs after several weeks of exposure to a xenobiotic within a certain concentration range. The motivation of the investigation should provide a historical and conceptual context in which the Cellular Diagnostic approach can operate. This is no different than the patient history that is essential to an accurate and confident medical diagnosis by the clinician.

The second component of this element is the *test evaluation*: this not only includes processing and assaying the samples, but also experimental design and statistical analysis. The act of collecting, processing and assaying the samples is the most visible component of Cellular Diagnostics. Designing the assay arrays and the competent execution of the assay protocols is only one aspect of the *test evaluation*. Appropriate experimental design and rigorous statistical methods are required to ensure validity of the data and that the data can be used as legitimate premises to infer logical conclusions. Hence, not only must there be a level of confidence in the accuracy and precision of the data, but there has to be a level of confidence in discriminating the differences between populations (reference vs. treatments). Only after acquiring an acceptable level of

confidence in both aspects of the test evaluation process can the clinician move to the third element of Cellular Diagnostics, the interpretative process.

The third element of the diagnostic process, reasoning and interpretation, can be divided into two different methodologies; mathematical theory-based methodology and an empirically-driven methodology⁹. The mathematical-theory methodology focuses upon symbolic logic and probability theory. Similar to recent developments in bioinformatics, the mathematical theory-based methodology uses methods of Bayesian analysis, neural networks, and fuzzy sets to create algorithms for pattern recognition and discrimination of biomarker data and to test (correlate) for relationships between biomarker pattern and qualitative categories of cellular conditions (*e.g.*, cellular resiliency vs. cellular stress vs. cellular degeneration). The empirical-based methodology is a five step process similar to that described by Ledley and Lusted (1959)⁹. The first step is establishing the question being asked concerning the cellular condition. For example, does exposure to a pesticide induce oxidative stress and to what extent? The second step lists the facts established from the test evaluation and the anamnesis. The third step is ranking or prioritizing the significance of the facts based on values perceived by the diagnostician. For example, the diagnostician may grade the accumulation of hydroxynonenal (HNE) in a pesticide-exposed population to be of great importance because of HNE's known role in cellular pathology and as a key indicator of oxidative damage. The fourth step is creating categories that qualify a cellular condition. In the context of oxidative stress, categories such as "nominal", "responding to an oxidative stress, but not exhibiting damage", and "exhibiting cellular damage" are crude but effective designations that provide a means to differentiate levels of physiological or process condition. The fifth step is the inclusionary and exclusionary categorization of the data with the appropriate cellular condition. These two methods are not exclusive of one another, but are rarely used in conjunction, probably because of the lack of available exposure and formal training to these two methods. Recent work with *in silico* cellular modeling using biomarker data from field-based studies may provide an effective solution in combining the two methodologies for a more robust diagnosis².

Strategy and Procedure

Cellular Diagnostics is a methodological tool that can be applied to almost any given situation in aquatic toxicology. The important question is: Is its application in a given investigation appropriate and correctly applied? One of the pillars of toxicology is the investigation of a dose response of an organism or cell to exposure of a xenobiotic. In keeping with the theme of this book, a strategy is described for using Cellular Diagnostics to examine the serial dose effects of a single xenobiotic to the hard coral species, *Porites porites*. The procedures needed to execute such an experimental design are also included.

Step 1 – Preparing the organism and experimental design

Materials-

Hammer or mallet
Steel or titanium leather punch
Metal, glass, plastic, or Teflon bolt with a head 1 to 2 cm in diameter
Marine epoxy or biological glue

Porites porites has a boulder-like coral structure that includes finger-like extensions from the main body. Unlike most branching coral species, (e.g., *Acroporids* and *Stylophora*) the coral tissue extends well into the coralline skeleton. Tissue with an abundance of zooxanthallae (the algal symbiont found in coral) can go as deep as 11 mm into the skeleton, and Coomassie Blue or Ponceau Red staining suggests coral tissue devoid of obvious zooxanthallae may extend 1-2 mm deeper. Coral plugs should be 1-2 cm in diameter, depending on how much tissue is needed for assaying, and should also be 2-4 mm deeper than the zooxanthallae-tissue zone. A steel leather punch is one of the best and most inexpensive tools for creating the initial coral plug. Steel leather punches can be obtained from any local hardware store. Care should be taken to ensure that the leather punch is rust free, and washed with soap (dish soap is best) of any oils or silicon grease before use. For the most part, the skeleton of most *Porites* species can easily be cored without much damage to the edge of the leather punch. Extended use or denser skeletons can damage the edge of the leather punch. Jagged or bent edges on a leather punch should be sharpened or, if beyond repair, the leather punch should be discarded.

Once the coral plug is made, it can be affixed to an appropriate mounting fixture such as the top of a clam shell, or alternatively, the top of a plastic or Teflon-coated bolt with a 1-2 cm diameter head is preferable (Figure 1).

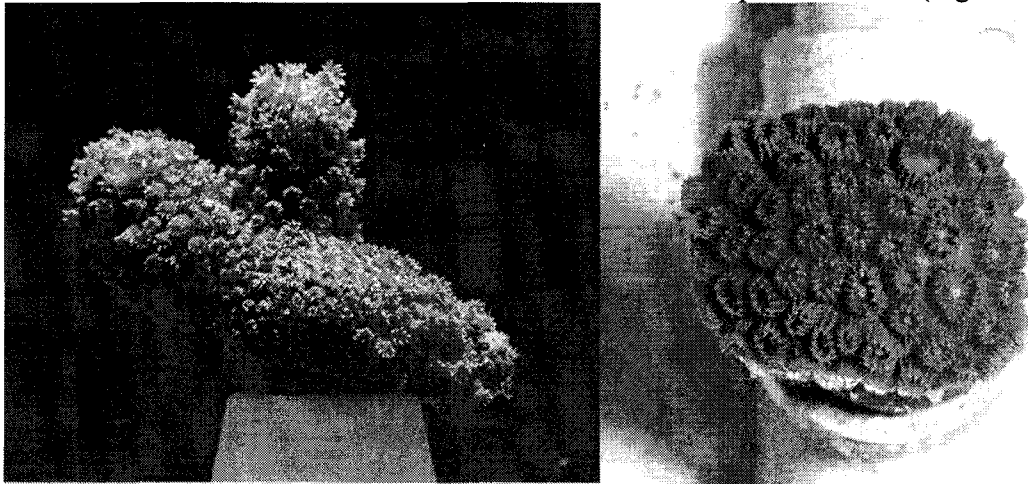


Figure 1 Coral plugs. Corals plugs can be mounted on an appropriate fixture so that they can be grown at high density and be easily handled. On the left, *Porites porites* coral plug in the branching form. On the right, *Porites porites* coral plug in a non-branching form. Photos courtesy of Tom Capo.

Using a bolt-like fixture will allow for higher densities of coral plugs to be placed in the same chamber, as well as ease of manipulating the plugs between chambers. The nature of the xenobiotic should be taken into consideration. If using a fuel/oil or other types of polycyclic aromatic hydrocarbons (PAHs) as a toxicant, Teflon-coated or solid-Teflon fixtures should be used because PAHs have a habit of adhering to “non-stick” materials,

altering the working concentrations of the xenobiotic in the dosing chambers. Care should also be taken in choosing the type of Teflon used. PFE or PFA Teflon is best and PTFE and related Teflons should be avoided because of their susceptibility to degradation in saline environments. There are several types of adhesives that can be used to affix the coral plugs to the fixture. Most workers use a marine epoxy (suggest Mr. Sticky's Underwater Glue (Fairbanks, California, USA) for its low toxicity) or a biological glue. Biological glues include byssal-thread glue or Collagen-based glues that are commonly used in the make-up industries that are saline resistant. Other glues that work well are medical glues such as Pros-Aide (ADM Tronics, Inc.) used in surgery or dentistry. It is important not to use standard marine epoxies because their potential toxicity, especially with prolonged exposures to ultra-violet light.

The act of creating a coral core induces a stress response. Cellular damage products such as lipid peroxides, malondialdehyde, hydroxynonenals, and protein carbonyl residues may take as long as 7-14 days to clear from the cellular system and return to basal levels. Chaperonins (e.g., Hsp60) and other stress-associated proteins may take as long as 21 days to return to basal levels. It is suggested that coral plugs be allowed to recuperate and acclimate for at least two months before being used in an experiment. Culture conditions like water flow, temperature, light, and feeding methods will not be discussed here, but the U.S. Coral Disease and Health Consortium can be used as a good resource for this information due to the participation of coral culturing experts from both commercial and academic sectors (http://www.coral.noaa.gov/coral_disease/cdhc.shtml).

The dosing system itself may be a significant source of artifact. Dosing corals in static chambers for even 24 hours can produce significant artifact. Most coral species require a specific rate of fluid flow over them. In some coral species, the optimal flow rate is almost negligible, while others the optimal rate may be as high as 4 mL/minute. *Porites porites* requires a high flow rate, and low flow rates can result in decreased growth rates, increased ammonia secretion, and a significant increase in many stress and growth proteins within 48 hours. Ammonia/nitrogen toxicity is an important factor to consider, especially with static dosing systems. Frequent water changes (e.g., every 12 hours) or a water flow-through system can control ammonia artifact effects. Light concentration is another significant source of artifact. Indoor ambient lighting is relatively low, even if it seems bright to our eyes (compare 50 micromoles of photons $\text{m}^{-1} \text{s}^{-1}$ in most laboratories versus 2100 micromoles of photons $\text{m}^{-1} \text{s}^{-1}$ outside at noon on a clear day). The rate of photosynthesis is dependent on the concentration rate of light, as are all of its potentially adverse by-products, such as active oxygen species. For photosynthetic organisms, many herbicides and toxicants are light-dependent for acute toxicity (e.g., methyl violagen, atrazine, diuron). At least 500 micromoles of photons $\text{m}^{-1} \text{s}^{-1}$ should be used during an experiment. Light quality is also another factor. Like plants, coral zooxanthallae require certain wavelengths of light more than other wavelengths to have optimal photosynthesis. The quality of light emitted by a sulfur lamp or a standard fluorescent lamp is not similar to the quality of natural light. Two options in resolving the issue of quality and quantity of light are to use natural light and alter the quantity of light by means of neutral density filters or to use a solar simulator generator. Though solar simulator generators can produce some of the most consistent light conditions from one experiment to another, their expense makes their use prohibitive.

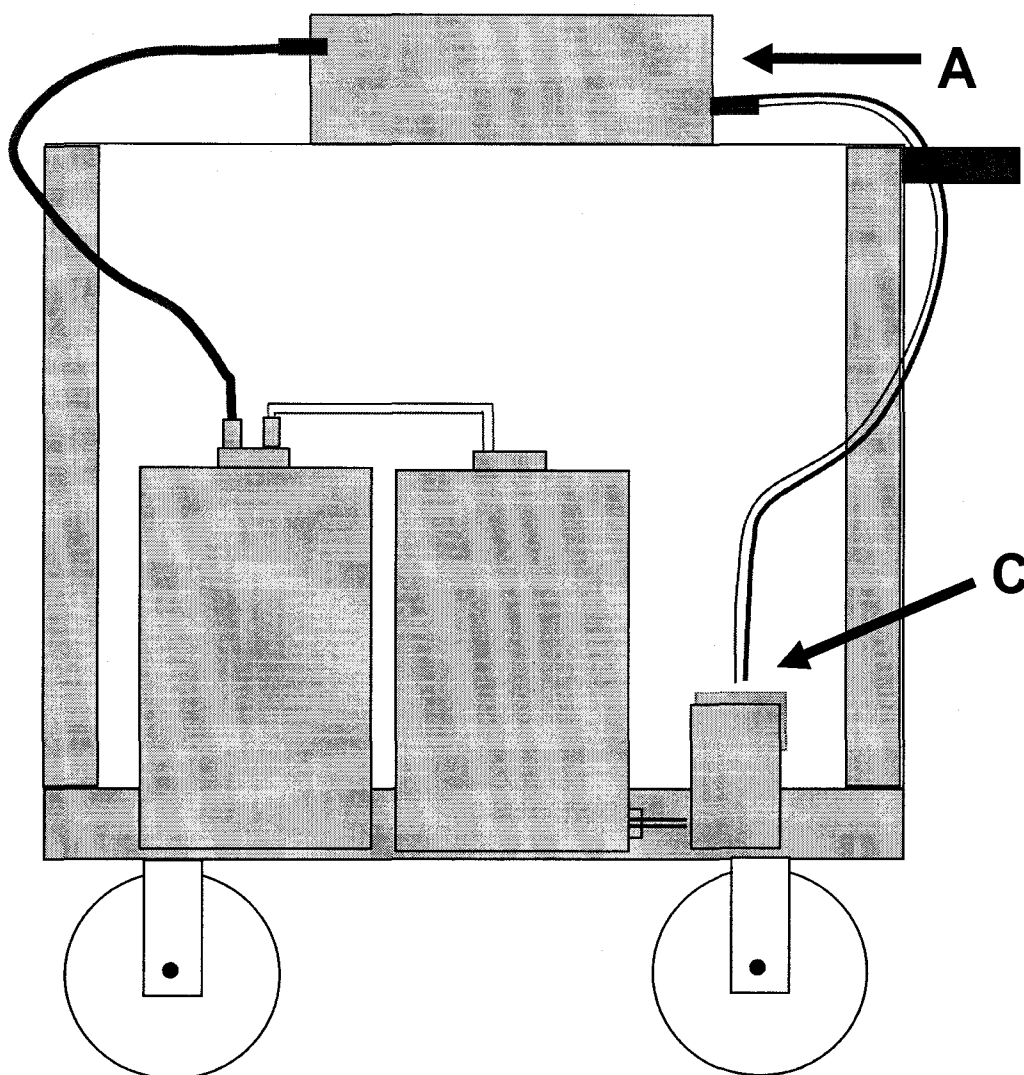


Figure 2 Dosing system for corals. Static systems can produce significant artifact, especially if the exposure to the xenobiotic is longer than 24 hours. The entire system can be placed on a two-shelf cart. On the top shelf is the dosing chamber, a chamber about 2 liters in volume, large enough so that the top of the coral plug is covered by at least 6 cm of water. The top of the dosing chamber is open to the air (light transmission). Water is pumped into the chamber from a set of 25 L reservoirs by a variable-flow pump that sits on the bottom shelf of the cart. Reservoirs can be changed out at designated time points (8-12 hours) with fresh solution of seawater/xenobiotic.

One of the best systems for coral dosing is a recirculating system (Figure 2). The system can be placed on a standard laboratory cart that has two benches. On the top bench sits an insulated dosing chamber that is deep enough so that the coral core is submerged at least 4 cm below the surface. On one end of the chamber is a flow-in port while on the opposite end of the chamber is the flow-exit port: seawater flows enters from the flow-in port and exists from the flow-exit port. On the bottom bench sits a 10-20 liter reservoir. A variable flow-rate pump will pump water from the reservoir to the chamber. Water flows back to the reservoir from the dosing chamber via the flow-exit port by gravity. Shut-off valves on the tubing that interfaces between the reservoir and

the dosing chamber can be installed to make it easy to switch out the reservoir at appropriate time intervals, depending on the length of the exposure. The top part or lid of the chamber can be left open to allow for lighting. A coil connected to a recirculating chiller/heater can be placed in the reservoir or dosing chamber to control for temperature.

Coral plug samples that are collected for testing should be rinsed free of excess mucus with fresh seawater, and quickly 'toweled' or dried with a fiber-free towel for excess seawater. Excess seawater with the samples can dilute the final concentration of the sample during sample preparation and can cause artifact when running the sample on a SDS-PAGE. Coral should be quickly frozen in liquid nitrogen or dry ice and kept at -80°C or colder until sample preparation.

Sample Preparation, Quality Control Validation, and ELISA protocol

Materials required

Equipment

- Ceramic or stainless steel mortar and pestle
- Microcentrifuge
- Vortex mixer
- pH meter
- Analytical balance
- Hot plate
- Container to boil water
- Polyacrylamide gel electrophoresis set
- Wet or semi-dry western transfer apparatus
- 96-well dot blotter or 48-well slot blotter
- Incubation chambers
- Rocker
- Micro-pipettors (10 uL, 200 uL, and 1000 uL)
- Multi-channel (10-200 uL) pipettor (8 or 12 tips placements)
- Scanner or camera imaging system
- Liquid nitrogen dewar
- Styrofoam box with lid (1 L)
- Computer

Supplies

- Locking micro-centrifuge tubes
- Cryovials (1.8 mL to 4 mL)
- Zinc-based spatulas
- Pyrex-based bottle (250 mL to 1000 mL)
- Micro-pipette tips (20 µL, 200 µl, and 1000 µL)
- Graduated cylinders (25 mL and 500 mL)
- PVDF membrane or nitrocellulose membrane with a pore size 0.2 microns or less

- Flat-plated forceps
- Micro-centrifuge tube holders
- Whatman filter paper No. 5

Reagents

- Liquid nitrogen
- Dry ice
- Distilled water or distilled/deionized water
- Trizma base (ultra pure grade)
- Sodium chloride
- Sodium dodecyl sulfate (ultra pure grade)
 - Note: use SDS from a bottle that has been opened for less than 45 days. SDS can oxidize over time reducing its properties and compromise the sample preparation
- Dithiothreitol (DDT) or B-mercaptoethanol (BME)
 - Note: DDT should be kept in the refrigerator and in a desiccator. When in solution, it should have a 'sweet' smell to it when wafted; if it has a "sour" smell to it, the DDT is oxidized and its capacity as a thiol reductant is severely reduced.
 - BME has a shelf life of about 60 days after initially opened, and will undergo considerable oxidation, reducing its effectiveness as a thiol reductant
- Disodium ethylenediaminetetraacetate (EDTA)
 - Note: EDTA will dissolve into solution if it is titrated with 5 M NaOH, final pH of the solution should be 8.0
 - Desferoximine methylate
- Phenylmethylsulfonylfluoride (PMSF)
- Aprotinin
- Leupeptin
- Bestatin
- Pepstatin A
- α -amino-caproic acid
- Salicylic acid
- Polyvinylpolypyrrolidone (PVPP)
- Dimethyl sulfoxide (DMSO)
- Coomassie Blue RR 250
- Methyl alcohol (methanol)
- Glacial acetic acid

Tests

The protocols described below focuses only on enzyme-linked immuno-sorbent assays (ELISA). There are a number of non-ELISA tests that can be included in a standard Cellular Diagnostic assay arrays. For example, spectrophotometric assays, such as those for lipid peroxide, glutathione, and for measuring specific enzyme activities (e.g.,

esterase, EROD) can readily be included in an array of tests with some modification of the sample preparation protocols. Other innovative assay protocols, such as in Chapter XX of this volume by Dr. Kouda and co-authors, PCR-based assays, COMET assay, HPLC-based assays can readily be included in a Cellular Diagnostic assay array.

Sample Preparation

Pre-chill the mortar and pestle with liquid nitrogen equivalent to two volumes of the mortar. Add half or all of a coral plug sample to the mortar. Immediately half-fill the mortar with liquid nitrogen, and after about five seconds begin grinding the sample with the pestle, making sure that the sample never thaws. The sample should be ground to a consistency between that of table salt and fine flour. The spatula and cryovial are pre-chilled in dry ice. Use the spatula to transfer the sample powder to the cryovial. The sample should be kept at -80°C or lower. Thawing and refreezing will cause the sample to solidify, making it impossible to use in further sample preparation steps unless it is reground.

The denaturing buffer should be made within four hours of use and consists of 2% SDS, 50 mM Tris-HCl (pH 6.8), 25 mM dithiothreitol, 10 mM EDTA, 0.001 mM sorbitol, 4% polyvinylpyrrolidone (wt/vol), 0.005 mM salicylic acid, 1% (v/v) DMSO, 50 µM desferoximine methylate, 0.04 mM Bestatin, 0.001 E-64, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamide, 5 µM α-amino-caproic acid,

and 1 µg/100 µL pepstatin A. Transfer 50-100 mg of sample from the cryovial to the locking-cap micro-centrifuge. Add 1400 µL of denaturing buffer to the micro-centrifuge tube, lock the cap to the tube, and vigorously vortex the sample for at least 10 seconds. Incubate the tube in a 90°C water bath for three minutes, vortex the tube for 10 seconds, and then

reincubate at 90°C for three minutes. Remove the tube and allow it to cool at room temperature for at least five minutes. Centrifuge the tube for 15 minutes at least 13,000 g. Three phases will be evident after centrifugation: the bottom phase is the insoluble phase consisting predominantly of coralline skeleton and PVPP (Figure 3). The middle

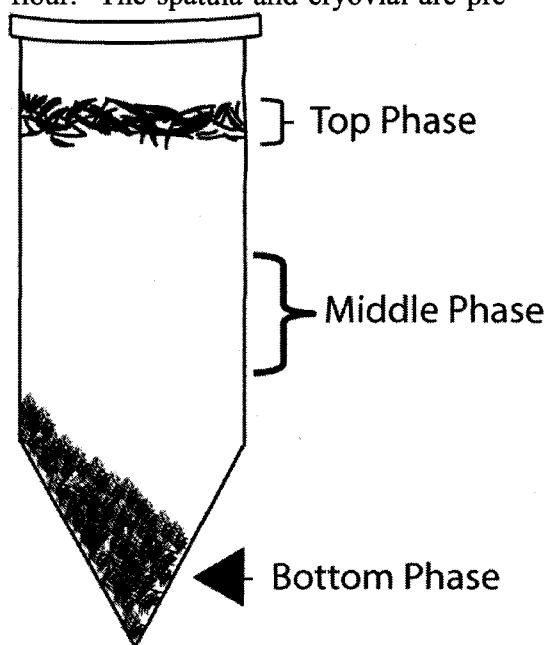


Figure 3. Sample preparation. Three phases are formed after centrifugation of the sample. The bottom phase is insoluble debris, primarily calcium carbonate skeleton and insoluble PVPP. The middle phase should be relatively transparent and should be absent of residual mucus. It should be non-viscous. The top phase should be somewhat viscous and sticky, whitish in color. The demarcation between the middle and top phase is not always apparent, and care should be taken that when aspirating the middle phase, there should be no contamination from the top-phase matrix.

phase should be transparent, brownish in color, and free of a whitish film that characterizes the top phase. The top phase is a very viscous matrix composed predominantly of cross-linked polysaccharide. Aspirate 200-300 μL of the middle phase; be careful not to collect any supernatant that may be contaminated with the whitish matrix that is the top phase. Deposit the middle-phase supernatant into a new tube.

Determining the protein concentration of the sample is necessary to equalize the sample for loading onto SDS-PAGE/western blotting assays, enzyme-linked immunosorbent assay (ELISA), and for normalizing the data when calculating the quantity of the parameter in the sample. Protein concentration is one of the most common parameters assayed, but unfortunately, it is one of the least understood assays. Besides sample preparation, assaying for protein concentration is a potential source of artifact and variation that can affect all subsequent assays. There are a number of good sources that explain both the method and theory of a number of different protein concentration assays that should be studied¹⁰. Many components of a denaturing buffer, as well as compounds inherent to the sample, may potentially interfere with a particular protein concentration assay. For example, chlorophylls and carotenoids in plant and photosynthetic material (e.g., coral, some species of gastropods and mollusks) can interfere with accurate measurements using Coomassie-based spectrophotometric assays. For corals, the Ghosh et al. 1988 protein concentration assay is a preferred method, because it is amendable to high-throughput, does not require a protein precipitation step to exclude interfering substances produced by the zooxanthallae, and is not affected by the harsh reagents found in the denaturing buffer recipe described in this chapter¹¹. Using filter paper Whatman No. 5 rather than using a microtiter plate is a better medium for a blotting platform. Using SDS denatured bovine serum albumin or oval albumin is acceptable protein concentration standards, though the highest concentration standard that should be used is 2.5 mg/mL. Sample protein concentrations above 2.5 mg/mL are suspect because the ratio of SDS to protein does not ensure adequate

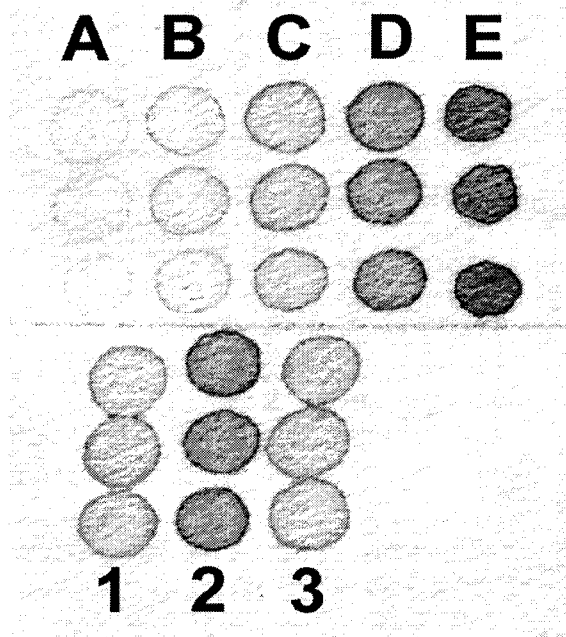


Figure 4 Protein Concentration Assay. Results of protein concentration assay using a modified method of Ghosh et al. (1988). Concentration calibrants are plated on the Whatman No. 5 filter paper as in Columns A - E. Column A = 0.125 $\mu\text{g}/\mu\text{L}$. Column B = 0.250 $\mu\text{g}/\mu\text{L}$. Column C = 0.5 $\mu\text{g}/\mu\text{L}$. Column D = 1.0 $\mu\text{g}/\mu\text{L}$. Column E = 2.0 $\mu\text{g}/\mu\text{L}$. Samples done in triplicate indicated in columns 1-3. All calibrants and samples are plated (hence assayed) on the filter paper in triplicate. Assay paper can be scanned by a scanner to create a digital image. Optical densities for each replicate can be determined using a densitometry program such as NIH Image developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>.

binding of the SDS to protein, which can lead to protein aggregation and precipitation as a result of freeze/thaws and during the heat-denaturation step of the sample preparation.

Apply 1 uL of sample in triplicate to the filter paper (5 or 10 cm diameter). Samples and calibrant curves can be designated on the paper using a pencil (No.2). Allow the samples to completely dry, either air dry for 10 minutes or with a hair dryer. Incubate the filter paper on a rocker in the Stain Solution for at least 10 minutes. Pour off the stain solution, and then apply 4-5 washes of the Destain Solution, incubating 5-8 minutes for each wash. The filter paper untouched by sample or calibrant should be white and not have any remnants of blue stain (4).

Stain Solution:

- 200 mL of distilled water
- 50 mL of glacial acetic acid
- 250 mL of methanol
- 4 g of Coomassie Blue RR 250

Destain Solution:

- 800 mL distilled water
- 200 mL of glacial acetic acid
- 1000 mL of methanol

Sample Validation

Quality Control Step 1 - Confidence that the assays are measuring the appropriate cellular marker is usually required for most scientific publications, government reports, or litigious discussions. SDS-PAGE/Western blot analysis is the minimum level of quality control/quality assurance. Western blot analysis will demonstrate that (1) preparation of the sample was satisfactory, and (2) that the antibody for the set of samples can be validly used for ELISA. Criteria for confidence assurance are:

- 1) There is no 'smearing' of the sample lane
- 2) Band(s) are present at the expected migration rate
- 3) Absence of non-specific cross-reactivity

Aliquots of 100 uL from four random samples should be pooled, the protein concentration determined for the pooled sample and applied to SDS-PAGE. Concentration of acrylamide and other gel contents are specific to the type of parameter being examined. For example, assaying of small heat-shock proteins and metallothioneins will require specific reductants in both the denaturing buffer and in the separating gel. Adding reductants, such as TCEP (pH neutral) at 1 mM in the separating gel, will ensure thiol reduction as the thiol rich proteins move through the gel. Altering the percent of acrylamide in the separating gel can enhance the resolution in seeing differences, depending on the molecular weight of the target protein. Finally, it is important to ensure that the gel has SDS in both the stacking and separating solution. Many pre-cast gels lack SDS, which can affect the migration rate of many of the target proteins.

Contents of gels are transferred to activated PVDF or nitrocellulose membrane using either a semi-dry or wet transfer system. PVDF blots are incubated in a blocking buffer and then transferred to the primary antibody solution for an allotted time at a specific temperature. Blots are washed four times with a wash buffer, and then incubated with an appropriate conjugated secondary antibody for one hour, washed four times with a wash buffer, then developed using an appropriate detection system. Replicates of the sample blots should also be assayed with only the conjugated secondary antibody with no exposure to a primary antibody in order to determine level of artifact caused by the conjugated secondary antibody (non-specific cross-reactivity).

Blocking Solution:

- 1x Tris buffered saline (pH 8.4); 500 mL; 15 mM NaCl, 50 mM Trizma base; titrate pH of buffer with 3 N HCl to 8.4
- 30 g of non-fat dry milk

Wash Solution

- 2x Tris buffered saline (pH 8.4)
 - Tween-20 0.01% (v/v). If the Tween is old (shelf-life of 45 days) it may undergo oxidation, causing an increase in non-specific cross-reactivity

Primary and Secondary Antibody Solution

- 2x Tris buffered Saline (pH 8.4)
- Pre-determine titer for optimal specificity

Blots can be developed either colorimetrically, chemiluminescently, or fluorimetrically. There are a number of good references that describe standard SDS-PAGE/western blotting procedures in detail^{10,12}.

The purified protein of the target parameter should be run on the lane next to the sample to ensure that the migration rates are the same. Using only molecular weight standards is a practical means of approximating that the band is the right migration rate, but there is more confidence in using the purified protein or a sample that you know has the protein, because you can account for the migration nuances of the protein running under specific buffer and gel conditions.

There are five types of results that can be observed on a blot when working with coral or other invertebrates that house an algal symbiont and plant samples. The five types of results are:

1. Band at the expected migration position with no other non-specific cross-reactive bands (Figure 5A)
2. Band is NOT at the expected migration position, but bands can be observed at different migration position. There is no confidence that the antibodies, the sample preparation or the running conditions are optimal for ELISA (Figure 5B)
3. Band at the expected migration position, but with bands at unexpected migration positions. For example, if you are assaying for invertebrate manganese superoxide dismutase, you should see a band around 25 Ku (standard international designation of kilo Dalton for gel electrophoresis).

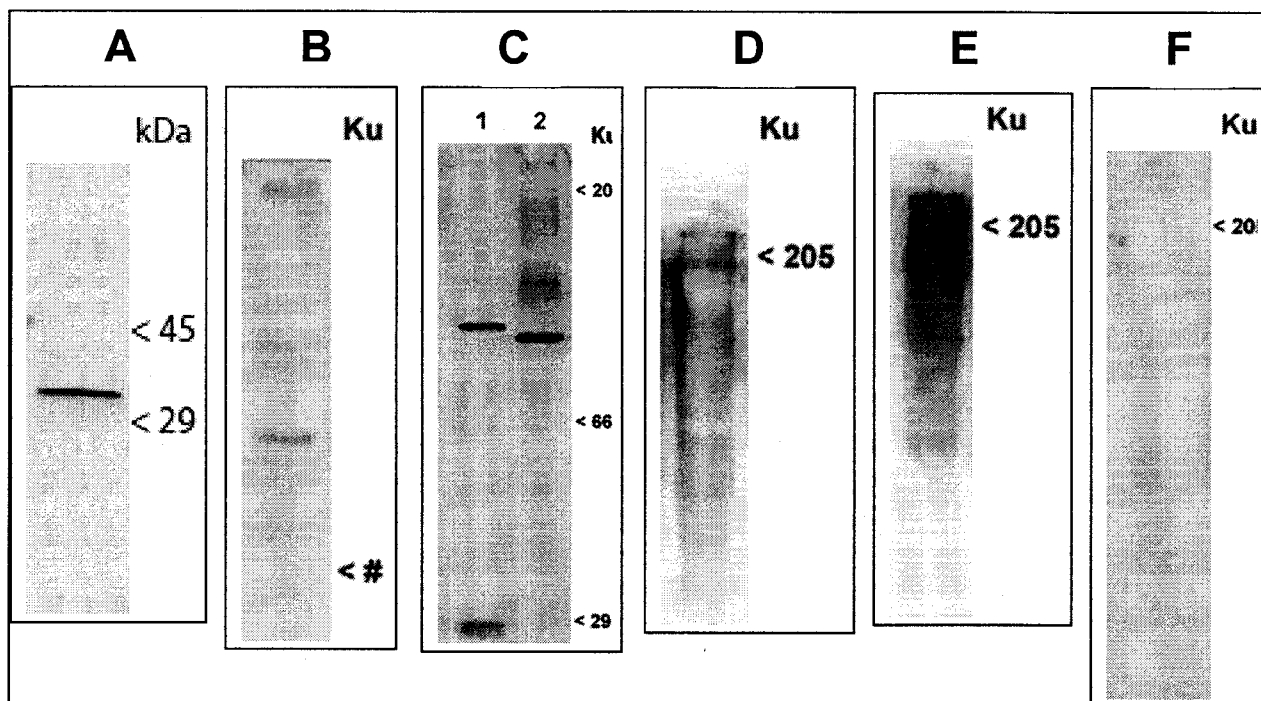


Figure 5 Quality Control Step 1: possible results for immuno-assaying with western blotting. Panel A – Acceptable result of a sample assayed on for heme oxygenase which should have a band around 32 Ku. Panel B – Unacceptable result of a sample assayed for glutathione-s-transferase which should have a band at the position designated by #. Panel C – Unacceptable result of a sample assayed for chloroplast small heat-shock protein (sHsp). In Lane 1, the monomer of the chloroplast sHsp is detected at the 29 Ku migration distance, but there are also higher-molecular weight bands. The sample in lane 1 was made up in sample buffer with 2% SDS, 10 mM thiol reductant, and was not heat-denatured. In lane 2, the sample was made up in sample buffer with 0.5% SDS, no thiol reductant, and was not heat-denatured. Notice that the monomer is not present. Panel D – coral sample (*Montastrea annularis*) that was prepared in a sample buffer lacking PVPP and exhibits streaking as a result of extensive Maillard Product contamination. Panel E – coral sample (*Montastrea faveloata*) that had extensive mucus contamination that was not removed during sampling. Panel F – coral sample that was assayed using only the HRP conjugated secondary antibody to ensure that the secondary antibody was not associated with any non-specific cross-reactivity.

You may also see bands around 40 Ku, and above the 190 Ku markers. There may be something wrong with the sample preparation, with the antibodies, or with the running conditions of the gel. For example, many enzymes exist in a multimeric structures, and require harsh denaturing and reducing conditions to break the enzyme complex apart into its separate components. These slower migrating bands may be the non-denatured, multimeric structures, as in the case of homogenization buffer lacking SDS and run on a native gel. In any case, there is no confidence in the sample or conditions to conduct an ELISA (Figure 5C)

4. There is a dark smear going the entire length of the sample lane. This is most likely due to extensive Maillard Product formation. Polyphenols from the algae are cross-linking proteins and carbohydrate. Increasing the concentration of PVPP or adding PVP (0.5% w/v maximum) may rectify this artifact. Additionally, Na tetraborate (5 mM maximum) may further

help reduce the level of Maillard Product formation. Nonetheless, there is no confidence that the samples can be subject to an ELISA (Figure 5D).

5. There is a dark smear running from the top of the lane to about the 66 Ku marker. This artifact is most likely the result of high polysaccharide contamination. When aspirating the middle phase during sample preparation, some of the top phase was also aspirated as part of the sample. Multiple centrifugations/aspirations, subjecting the sample to a polysulfone micro-centrifugation filter, or increasing the level of DMSO or adding L-cysteine (1 mM maximum) may help to reduce this artifact. There is no confidence that the samples can be subject to an ELISA under their present state (Figure 5E).

When assaying a sample blot with only the secondary antibody, the blot should be clear. Any bands or smears appearing on the blot can mean that the secondary antibody used for both western assay and the ELISA is non-specifically cross-reacting with some epitope in the sample (Figure 5F). Fab fragments conjugated with the appropriate reporter enzyme usually give the best results under most situations¹³.

Quality Control Step 2 and ELISA optimization - The pooled sample from Step 1 is serially diluted eight-fold, the highest concentration is 6.667 ng/μL. A 16-fold serial dilution of the calibration standard is also created. The calibration standard is the purified protein or antigen being assayed. The concentration of the calibration standard should be known and designated either in moles or grams of the antigen. Both the sample serial dilution and the calibrant serial dilution should be plated on a 384-well microtiter plate or on a membrane dot or slot blotter. Because most labs can affordably access a dot or slot blotter, the protocol will focus exclusively on using a dot/slot blotter for carrying out the ELISA. If using a Bio-Rad or other brand of dot/slot blotter, activate the PVDF membrane in methanol, and then equilibrate the membrane in 1x TBS for 1 minute. If using a nitrocellulose membrane, equilibrate the membrane for 1 minute in 1x TBS. Quickly place the membrane within the manifold of the dot/slot blotter, and assemble the dot/slot blotter. Apply the serial dilutions of both the sample and the calibrant in triplicate on the dot/slot blotter. Contrary to most manufacturers' instructions, do not incubate the serial dilutions on the dot/slot blotter for 20 minutes or longer. Once the samples and calibrants are loaded onto the blotter, suctioning the samples through the membrane should be done immediately. A hand-held vacuum, such as the Nalgene Hand Pump is one of the best instruments for creating a vacuum for a dot/slot blotter. You can control the vacuum pressure much more readily than with a motorized pump, as well as better control in releasing the vacuum. A slow release of the vacuum pressure is best because it prevents backwash of the samples back across the membrane; a major source of artifact for dot/slot blotters. Immediately transfer the membrane to the blocking solution, and carry out the development of the blot using the same protocols as done in Quality Control Step 1.

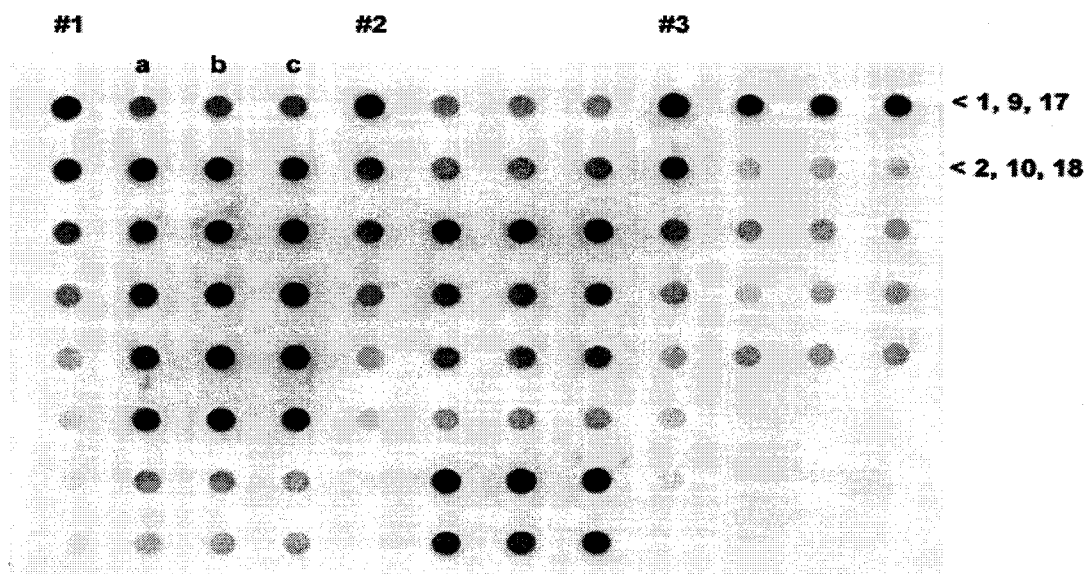
Once the blot is developed, scan or image-capture the blot. NIH Image is an easy to use and free optical density program that can be used on PC or Macintosh computers. Determine the optical density of each point, and then plot the data. A trend line can be fitted using either a liner or polynomial (second order) regression, and the equation and R

square for the trend lines determined¹³. Lack of linearity (or curvilinearity), such as a low R^2 value is an indication of artifact¹³. Sample loading, primary and secondary antibody titers, as well as incubation and wash conditions can all be optimized during this step. Intra-specific variation for each triplicate concentration point should be less than 15%. Finally, the calibrant standard curve should be optimized with the sample loading so that the signal of the sample falls somewhere in the middle of the calibrant standard curve. To increase the probability that all the samples will fall within the calibrant standard curve, create a serial dilution of a sample from the reference treatment and a serial dilution of a sample from the highest concentration from the xenobiotic-exposure treatment.

A third Quality Control Step is optional, but bestows the highest confidence that the antibody is truly detecting the correct protein or antigen in the samples. This step entails the construction of an immuno-column using the primary antibody, purification of the immuno-column elutant, extensive fractionation protocols if there are more than one protein eluted from the columns, and Edman degradation or Mass spectrometry peptide sequencing. This step is optional only because of the expense, extensive training, and accessibility to expensive supplies and equipment that is required to conduct the Quality Control Step. Protocols for this step can be found in a number of good resources¹⁰

ELISA - All samples and calibrants should be plated onto the dot/slot blotter in triplicate. Optimally, a single calibrant curve should be plated to the left, middle, and the

Figure 6 ELISA result using a 96-well dot blotter. # = calibrant standard curve. The calibrant with the highest concentration is plated in row 1, the calibrant with the lowest concentration is plated in row 8. Optimally, replicates of the standard curve should be evenly distributed across the dot blotter. In row 1, triplicate assays of sample 1, 9, 17. In row 2, triplicate assays of sample 2, 10, 19. In row 3, etc. In column *a*, replicate one of sample 1, 2, 3, etc... are plated. In column *b*, replicate two of samples 1, 2, 3, etc... are plated. In column *c*, replicate three of samples 1, 2, 3, etc... are plated. Plate represents data for the chloroplast small heat-shock protein in samples of the hard coral, *Montastraea annularis*.



right sides of the dot/slot blotter (Figure 6). Sample replicates can be plated in a similar fashion, or plated close together. The reason for dispersing the calibrant curves

throughout the micro-titer plate is to account for the potential of edge effects or differential staining from one side of the blot to the other. Data should be calculated as mole or gram per nanogram of total soluble protein (normalization). For example, when measuring Hsp60, the data should be calculated as moles of Hsp60/ng total soluble protein or grams of Hsp60/ng total soluble protein.

Statistical Analysis of Data

The first step of the analysis is to determine if the level of a specific parameter in any of the treatments are significantly different from the control population. Data should be first tested for normality and equal variance. If the data are normally distributed, apply a one-way ANOVA, but if the are not normally distributed consider the assumptions of other types of analyses, such as a one-way ANOVA on Ranks (*e.g.*, WELCH ANOVA). If treatment differences are found, apply the appropriate planned comparison test¹⁴ (Figure 7).

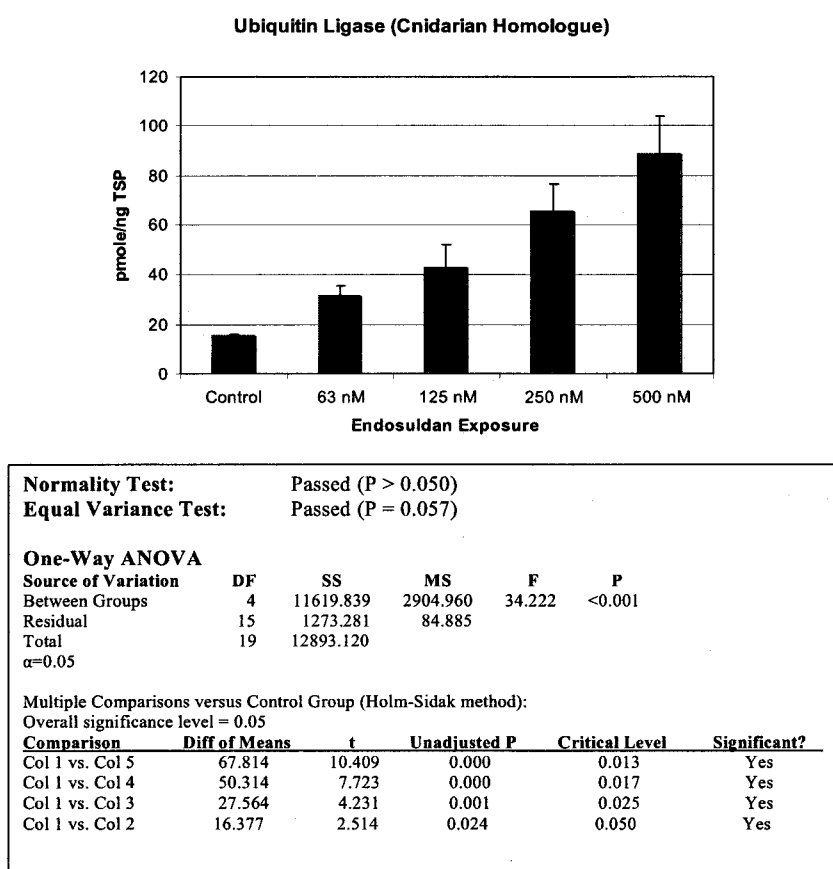


Figure 7 *Porites lobata* (tropical hard coral species) exposed to endosulfan (a pesticide) in a dose response experimental design. N = 4 per treatment. Coral plugs were exposed to differing concentrations of endosulfan for 12 hours, then prepared and assayed using the protocol described in the chapter. Top panel is the data for the cnidarian homologue of ubiquitin ligase (E2), an enzyme that conjugated ubiquitin to a protein slated for complete proteolysis. Bottom panel is the statistical method used to test for differences in protein levels of ubiquitin ligase between the different treatments and the reference control.

Canonical correlation analysis (CCA) has been used in the past as a heuristic tool to illustrate how biomarkers can be used to discriminate among xenobiotic and environmental stressors^{15, 16}. CCA is an Eigen analysis method that reveals the basic relationships between two matrices, in this case those of xenobiotic treatments and biomarker data. The CCA provides an objective statistical tool for determining which biomarker (or suite of biomarkers) best reveals the presence or response of a particular xenobiotic and environmental stressor. In the case of discriminating the effects of two or more xenobiotic/environmental exposures, two assumptions of CCA are made that result from the experimental design used; (1) that stressor gradients are independent and linear and (2) that biomarker responses are linear (Figure 8).

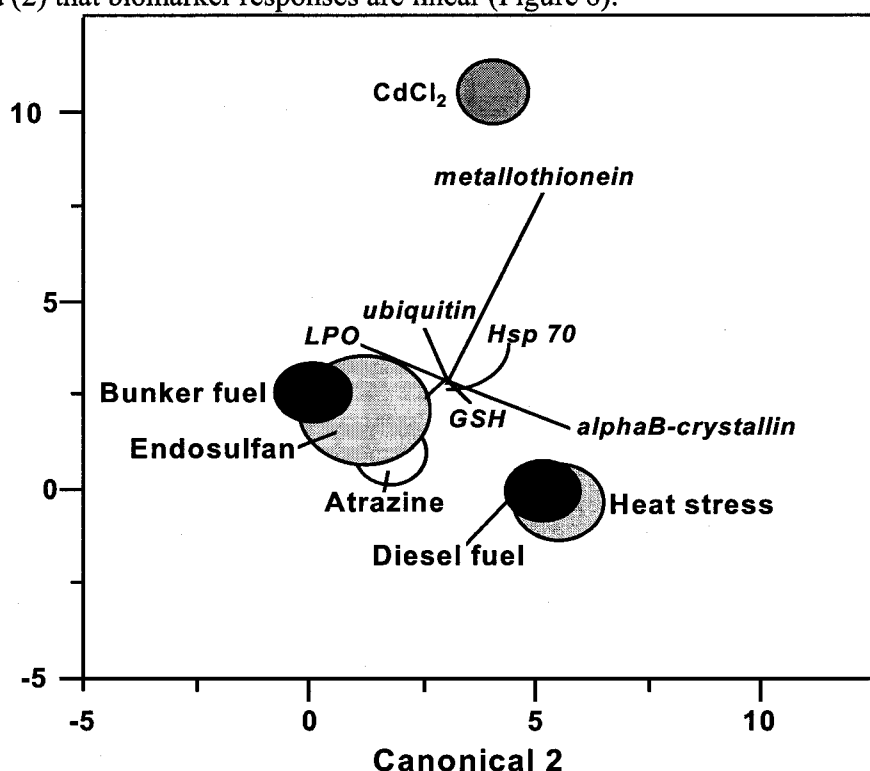


Figure 8 Canonical centroid plot of standardized biomarker responses in an organism exposed to a pesticide, an herbicide, a heavy metal, heat stress, and two different types of fuel. Original variates were biomarker levels expressed as a percentage of the control value in each experiment. Circles show the 95% confidence intervals around the distribution centroid of each stressor. Biplot rays radiating from the grand mean show directions of original biomarker responses in canonical space. Metallothionein, which responds to heavy metal stress, distinguished the cadmium chloride treatments from all others; α B-crystallin distinguished diesel fuel and heat stress versus the others.

Canonical correlation analysis can discriminate between the effects of the different concentration doses using the same protocol as above, but CCA can also differentiate the patterns of system resiliency and system degeneration. Instead of randomly choosing or compiling all the biomarker data into a single matrix, biomarkers from only a specific Cellular Diagnostic category are included into the matrix. For example, only parameters of protein metabolic condition are included into one matrix

while the other matrix is the reference and xenobiotic treatments compose the second matrix (Figure 9).

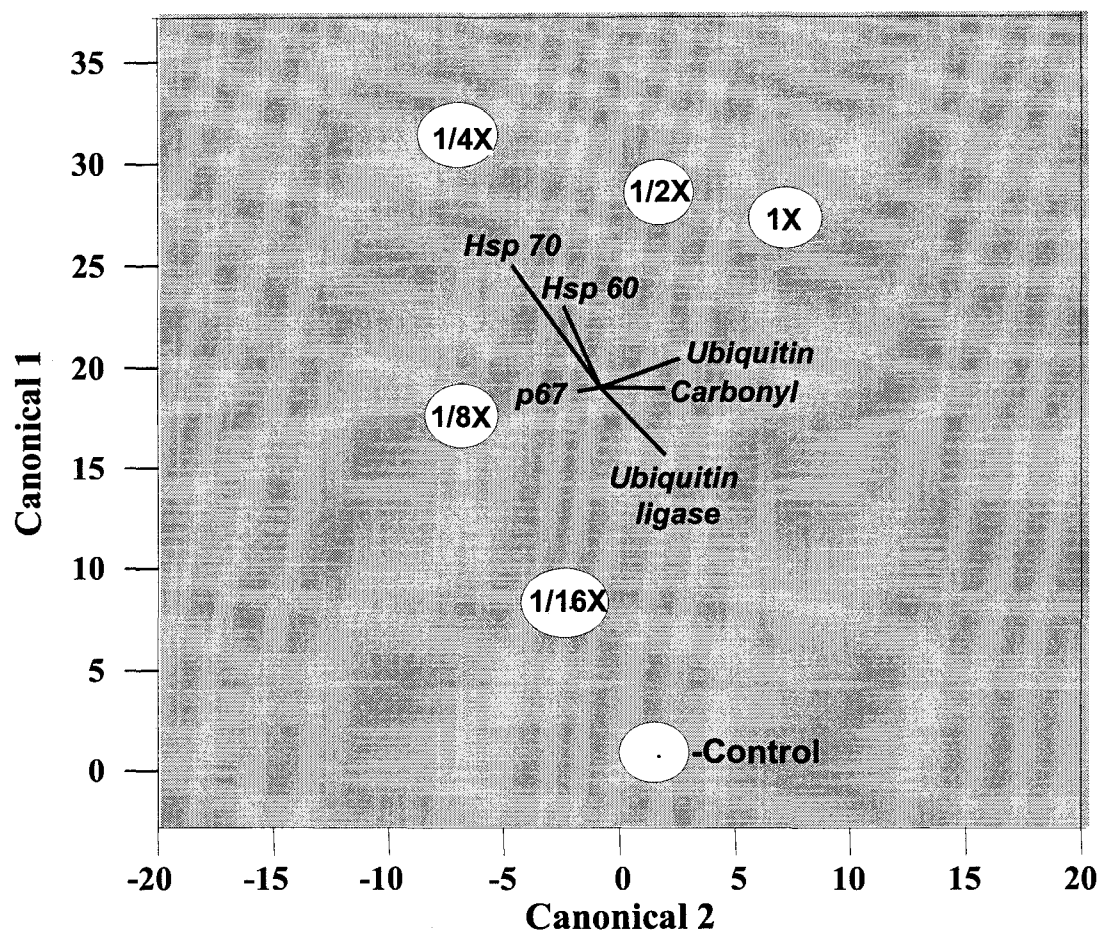


Figure 3 Canonical centroid plot using six parameters of protein metabolic condition in liver cells exposed to the different concentrations of the hepatotoxicant, thioacetimide. Exposure to thioacetimide was 24 hours. Concentration of thioacetimide is: 1 mM at centroid plot 1X, 500 μ M at centroid plot 1/2X, 250 μ M at centroid plot 1/4X, 125 μ M at centroid plot 1/8X, and 63 μ M at centroid plot 1/16X. Biplot rays radiating from the grand mean show directions of original biomarker responses in canonical space. Notice that protein oxidative damage product (protein carbonyl) and a marker for protein degradation (ubiquitin) define differences between the 1x centroid and the control, indicating that at 1 mM thioacetimide induces protein oxidation (oxidative stress) and increase in the rate of protein degradation.

Table 1 Categories of Cellular Integrity and Homeostasis

- **Genomic integrity** – the ability of the genomic process to maintain a functional state. Some parameters that are used as an index for this condition include DNA damage products (e.g., xenobiotic adducted to DNA, oxidized DNA, and DNA abasic sites), DNA repair systems (e.g., DNA glycosylases), occurrence of single and double strand breaks, chromosomal response, and mutation.
- **Protein metabolic condition** – the process of protein synthesis, protein maturation, and protein degradation. Parameters that are sensitive to changes in this category include p67, concentration and activation state of various Hsp90s, Hsp60, grp78, ubiquitin, LON proteases, and specific ubiquitin ligases.
- **Xenobiotic detoxification** – the process of preventing or reducing the adverse (toxic) effects of exposure to a xenobiotic. Some parameters of the detoxification condition of the cell include concentration and activity of various cytochrome P450 monooxygenase, glutathione-s-transferases, and members of the ABC protein family (P-glycoprotein 180/MDR).
- **Metabolic integrity** – the process of a cell in maintaining a differentiated state from its environment and is the product of sub-processes or 'metabolic' pathways. Parameters include the concentration and activity of specific enzymes (e.g., aconitase, Pepcase, ferrochelatase) and discrete organic products (e.g., pyruvic acid, ATP).
- **Oxidative damage and response** - the process of maintaining a viable condition in an oxygen-laden environment. Parameters include changes in the concentration of oxidative damage products (e.g., protein carbonyl groups, oxidized DNA, aldehyde) and anti-oxidant enzymes and solutes (e.g., catalase and sorbitol).
- **Immuno-competence** - the process of defending against microbial invasion. Parameters include anti-microbial peptides (e.g., defensins), sterols, sulfonated glycolids, and polyphenols.
- **Autophagy** – the process of removing organelles and other cellular structures by self digestion. This includes sub-processes such as eradication of mitochondria via lysosomal digestion and micro-nuclei deletion. Parameters that reflect autophagy state include dye retention of lysosomes (e.g., neutral red assay), activation state of Apg16p, Apg12p, and ceramide content.
- **Endocrine competence** – the ability of the cell to communicate with other cells to coordinate phenotypic expression and tissue function. This includes processes of differentiation, inflammation, and stress tolerance.
- **Membrane integrity** – the purpose of a membrane is to act as a selectively permeable barrier. Components and activities that can be used as an index for membrane condition include lipid peroxide and peroxidation breakdown products, sterol content and composition, fatty acid composition, and integral and peripheral membrane protein complexes.

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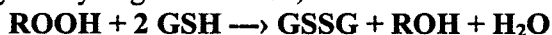
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Partial Listing of Cellular Parameters Used in CDS and their Biological Significance

Copper/Zinc Superoxide dismutase (Cu/Zn SOD) – Superoxide dismutases catalyze the reaction of superoxide ions and two protons to form hydrogen peroxide and O₂ (Fridovich, 1995). Copper/zinc SOD is found only in the cytosol of animal cells while homologues of this enzyme are found in both the cytosol and chloroplast in plants and algae (Fridovich, 1995). It is expressed constitutively, but levels increase in response to an increase of reactive oxygen species.

Manganese superoxide dismutase (Mn SOD) Manganese SOD is localized to the mitochondria in eukaryotic cells (Fridovich, 1995). Superoxide dismutases accumulate in response to oxidative stress and are one of the main anti-oxidant defense pathways. Increased levels of Mn SOD have been linked to increased longevity, increased tolerance to ischemic/reperfusion events, and increased tolerance to factors that induce oxidative stress (Fridovich, 1995). 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 (Fridovich, 1995).

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,



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. Selenium concentrations regulate GPx activity, and 4-hydroxynonenal (a lipid peroxidation product) can inhibit GPx, thus increasing the oxidative burden of cells. 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 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 (Seis, 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 (De Zwart et al., 1999).

Heat-shock protein 60 & 70 (Hsp60 & 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 chaperones 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.

Metallothioneine – Metallothioneines are cysteine-rich, low-molecular-weight proteins that will bind a variety of metals depending on the class of metallothionein (for review, Klassen et al., 1999). *In vitro* evidence demonstrates that metallothionein may act as an anti-oxidant, a factor to ensure a reservoir of zinc, and can protect against metal toxicity (Klassen et al., 1999). *In vivo* evidence using transgenic animals show only strong support for its function as a protectant against metal toxicity. Metallothioneins can be grouped into three classes (MTI, MTII, MTIII) (Klassen et al., 1999). In this project, we measure total levels of metallothionein MTI. Metallothioneins are strongly induced by metal toxicity and are indicators of exposure of toxic levels of some metals.

Ferrochelatase – Ferrochelatase is an enzyme that inserts ferrous iron into protoporphyrin IX to form heme. In invertebrates, this enzyme is able to utilize a number of different metals and porphyrin substrates. Both cellular detoxification pathways and essential cellular metabolism require heme or porphyrin-based substrates. For example, cytochrome *c* uses a form of heme in order to become an active electron carrier. Further, the class of monooxygenases, cytochrome P450, requires heme to function. If an organism is going to up-regulate metabolic or xenobiotic detoxification pathways, it will need to increase its heme production, thus, it will need to up-regulate ferrochelatase. Therefore, changes in ferrochelatase levels are indicative of changes in metabolic states, usually in response to a stress. Finally, the breakdown product of heme leads to the formation of bilirubin and biliverdin. Bilirubin is a very effective anti-oxidant, rivaling vitamin E as a scavenger of lipid hydroperoxyls.

P-Glycoproteins 160 & 180 (MXR or Multixenobiotic Resistance Protein) P-glycoprotein plays a role in xenobiotic detoxification. Currently, it is believed that P-glycoprotein effectively processes certain xenobiotics 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.

Invertebrate small heat-shock proteins (sHsp = total small heat-shock protein isoforms)
– α B-crystallin, Hsp22, Hsp23, Hsp26, and Hsp28 share domains of common

homology to one another, but have different cellular functions (de Jong et al., 1993). In cnidarians, as many as 5-6 major sHsp isoforms exist. 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.

α B-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). Evidence indicates that Hsp22 localizes to neural-type cells and follicular cells in *Drosophila melanogaster* (Downs, Towers, Downs, Woodley, unpublished data). Furthermore, this protein localizes to the mitochondria in arthropods and has been suggested to have a functional role similar to that of plant and mammalian mitochondrial sHsps. 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, chlp sHsp is found only in plant chloroplasts. This protein functions to protect Photosystem II activity, specifically the Oxygen Evolving Complex, during photooxidative conditions (Downs et al., 1999a, b; Heckathorn et al., 1998). This protein is not normally present during non-stressed conditions, but is induced by heat stress, high-light stress, UV stress, dehydration and during exposure to some herbicides (e.g., atrazine). Most likely it functions via a recycling anti-oxidant mechanism (Downs et al., 1999). The presence and concentration of these different small heat-shock proteins provides an index for the physiological status of a number of different metabolic pathways in the cell.

Heme Oxygenase – Also known as Hsp32, is up-regulated in response to oxidative damage. Heme oxygenase degrades heme to biliverdin, free iron, and carbon monoxide. Improper regulation of this enzyme is associated with a number of diseases, such as sporadic Alzheimer disease. An NADPH-dependent catalytic step converts biliverdin to bilirubin, a powerful anti-oxidant.

Ubiquitin – Ubiquitin is a 76-residue protein found in most phyla of life and used in a process for marking proteins for rapid degradation (Hershko and Ciechanover, 1998). Ubiquitinated proteins are degraded by proteolytic enzymes known as proteasomes (Hershko and Ciechanover, 1998). Proteins, during stress, are targeted for degradation usually because these proteins have undergone an irreversible denaturation (Iwai, 1999). Increases in ubiquitin levels are an indication of increased levels of protein degradation, and hence, increased protein turnover (Goff et al., 1988). Consequently, to compensate for decreased functional protein levels due to stress, the cell will increase production of these same proteins (Jennissen, 1995; Iwai, 1999). Thus, measurement of levels of ubiquitin is an index of the structural integrity of the protein component of the superstructure of the cell (Mimnaugh et al., 1999). Increased ubiquitin levels indicates: (1) a protein denaturing stress is occurring; (2)

increased expenditure of energy is required to compensate for this stressed-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 (Hawkins, 1991).

Cytochrome P450 2E homologue (CYP P450) –Cytochrome P450 2E is known to specifically oxidize ethanol to acetaldehyde via a monooxygenase mechanism, as well as other xenobiotics such as imidazole-based derivatives (Lieber, 1997). Cytochrome P450 2E has both physiologically relevant oxidative and reductive reactions and is known to associate and catalyze as many as 60 xenobiotic-based substrates (Koop, 1992; Lieber, 1997). For example, it causes the demethylation of N, N-dimethylnitrosamine and the hydroxylation of p-nitrophenol and chlorzoxazone (Koop, 1992). Reduction reactions include reduction of a number of different lipid types. One of the primary reasons for using the 2E class of cytochrome P450s is that it is not induced by heat stress, but can respond to hypoxia/reperfusion events in mammals (Bayanov & Brunt, 1999; Downs et al., in press).

Physiological Parameter

Benzodiazepene Receptor.
Carbonyl
Catalase
Chlp sHsp
Cu/ZnSOD Cnidarian
Cu/ZnSOD Dino
CYP P450-2 class
CYP P450 3-class
CYP P450 6-class
Ferrochelataase
GPx Cnidarian
GPx Dino
Grp75
GST Cnidarian
GST Dino
GST-pi
Heme Oxygenase
Hsp 90
Hsp60 Cnidarian
Hsp60 Dino
Hsp70 Cnidarian
Hsp70 Dino
Multidrug Resistance Protein (MDR)
Metallothionine
MnSOD Cnidarian
MnSOD Dino
8-oxoguanine DNA glycosylase (Ogg1-nuclear)
PBRP
Protoporphyrin Oxidase
sHsp
sHsp Cnidarian
Ubiquitin Activase
Ubiquitin

Classification

Steroidogenesis and inflammation
Oxidative Stress
Oxidative Stress
Metabolic Condition
Oxidative Stress
Oxidative Stress
Xenobiotic detoxification & endocrine modulation
Xenobiotic detoxification & endocrine modulation
Xenobiotic detoxification
Metabolic Condition
Oxidative Stress
Oxidative Stress
Protein Metabolic Condition
Xenobiotic Response
Xenobiotic Response
Xenobiotic Response & Oxidative Stress
Oxidative Stress
Protein Metabolic Condition
Protein Metabolic Condition
Protein Metabolic Condition
Protein Metabolic Condition
Protein Metabolic Condition
Xenobiotic Response
Metabolic Condition & metal metabolism
Oxidative Stress
Oxidative Stress
DNA Damage
Steroidogenesis and inflammation
Metabolic Condition
Metabolic Condition
Metabolic Condition
Protein Metabolic Condition
Protein Metabolic Condition

A novel diagnostic system to assess the physiological status of corals

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Abstract We developed a novel molecular diagnostic system to assess the physiological status of corals by assaying specific parameters of cellular physiology. This system is particularly relevant because multiple environmental stressors may contribute to bleaching, one mechanism for coral mortality worldwide. In laboratory studies, diagnostic parameters distinguished the separate and combined effects of heat and light on a star coral (*Montastraea faveolata*) and its symbiont (zooxanthellae), and provided evidence that oxidative stress plays a central role in bleaching. We also used this new biotechnology to characterize bacteria-induced bleaching in the ivory tree coral (*Oculina varicosa*). Our data suggest that bacteria-induced coral bleaching may involve non-oxidative stress mechanisms. Thus, the molecular diagnostic system enabled us to (1) diagnose whether corals were physiologically stressed, (2) potentially discriminate between mechanisms involved in coral bleaching, and (3) provide evidence that chloroplast small heat-shock proteins protect against coral bleaching.

Key words Coral bleaching, Molecular biomarkers, Health assessment, Stress proteins

Introduction

We designed an integrated molecular diagnostic system (MDS) that focuses a comprehensive array of diagnostic tools from biomedical research on environmental health issues (Downs et al. 2000, 2001). The MDS is an array of biomarkers of known biochemical, cellular and physiological processes and are tailored to individual species and likely sources of environmental stress. The system was designed with redundancy; several parameters from differing biochemical pathways characterize physiological status, thereby allowing concordance among markers for a more reliable diagnosis. The MDS assays specific cellular parameters such as lipid peroxide levels (LPO), malondialdehyde (MDA), 4-hydroxy-2-trans-

nonenol (HNE), total glutathione (GSH), heat-shock proteins 60 and 70 (Hsp60, Hsp70), α B-crystallin homologue, chloroplast small heat-shock protein (chhpsHsp), homologues to the plant class I-IV small heat-shock proteins (sHsps), Mn and Cu/Zn superoxide dismutases (MnSOD and Cu/ZnSOD), metallothioneins, specific P450 family members, protein carbonyl and ubiquitin. These parameters indicate the physiological condition of critical components of the cellular superstructure (i.e., enzymes, DNA, membranes and metabolic pathways) and the status of general properties of cellular metabolism (i.e., photosynthetic and oxidative phosphorylation). The MDS also quantifies responses to oxidative stress and provides evidence for putative stressor identification (i.e., heat, pathogens). Together, these physiological measurements diagnose organismal health by coupling an understanding of cellular physiological responses with organismal fitness.

We used the MDS to investigate a serious environmental health issue: the extensive worldwide degradation of coral reefs (Dustan 1999; Stone et al. 1999). Coral bleaching is a major contributor to reef declines. Bleaching is a process whereby corals lose their obligate symbiotic algae (zooxanthellae) and/or the symbionts' photosynthetic pigments become degraded (Gates et al. 1992; Brown 1997a). Although elevated seawater temperatures (i.e., heat stress) are a primary factor in most large scale coral bleaching events (Hoegh-Guldberg and Smith 1989; Lesser et al. 1990; Glynn 1993; Goreau et al. 1992; Goreau and Hayes 1994; Lesser 1996; Brown 1997b; Hoegh-Guldberg 1999), other environmental factors also have been linked to coral bleaching, including cold stress (Coles and Fadlallah 1991; Muscatine et al. 1991), elevated visible light (Hoegh-Guldberg and Smith 1989), light deprivation (Yonge and Nichols 1931), ultraviolet light exposure (Kinzie 1993; Lesser et al. 1990), low salinity (Fang et al. 1995; Goreau 1964), starvation (Yonge and Nicholls 1931) and bacterial infection (Kushmaro et al. 1996; Ben-Haim et al. 1999).

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Several stressors may induce coral bleaching, so it is essential to determine the underlying mechanism(s) to manage or control it. Research has only recently focused on cellular and molecular aspects of coral bleaching (Black et al. 1995; Fang et al. 1997; Jones et al. 1998; Moshe et al. 1999; Downs et al. 2000). Here we present data from a new biotechnology that determines the cellular physiological status of corals. In laboratory studies, we tested whether the MDS distinguished the separate and combined effects of high temperature and light stress on a star coral (*Montastraea faveolata*). These two factors are strongly correlated with coral bleaching (Downs et al. 2000). We also used the MDS to test whether the biomarker parameters associated with bleaching induced by a pathogen (*Vibrio shiloi*) in *Oculina varicosa* differed from those expressed during high temperature stress.

Materials and Methods

Heat stress treatment

Skirt fragments from three colonies of *M. faveolata* were collected near Big Pine Key, FL (depth = 6 m) for laboratory heat stress experiments. Explants were maintained in aquaria at 27° C under an irradiance of ~350 $\mu\text{moles photons m}^{-2} \text{ s}^{-1}$ (12 h light: 12 h dark), and fed *Artemia* nauplii once per week.

Coral explants were subjected to three treatments (1) optimal growth conditions, (2) high temperature stress in the absence of light, and (3) high temperature stress under nominal photosynthetically active radiation (PAR), as described in Downs et al. (2000). Control samples were removed from optimal growth conditions and frozen. Heat-stress experiments were conducted in the light and dark by ramping the temperature from 27° - 33° C over 3 h and maintaining it at 33° C for 9 h. Each treatment was replicated three times and all samples were frozen at the end of the experiment.

Bacterial infection

One colony of *O. varicosa* was collected from Caper's Reef (depth = 18 m) near Charleston, SC for use in laboratory infection studies. Coral explants were maintained in aquaria at 26° C under 55 W blue ultra-actinic light (Custom SeaLife Inc.) on a 12 h light: 12 h dark cycle and fed *Artemia* nauplii once per week.

A single colony of *V. shiloi* was used to inoculate 25 mL of marine broth containing tryptone (MBT) media and grown at 30°C with orbital shaking (200 rpm) for 19 h. The inoculum was prepared by centrifuging 10 mL of the culture for 10 min at 9680 xg and washing the cell pellet twice in filter sterilized seawater. The final pellet was then resuspended in 1 mL of sterile seawater. Coral explants were acclimated in 1L dosing chambers adjusted to 28.5 - 29°C for one week prior to inoculation. Explants were infected by placing them in a sterile petri dish and applying 20 μL of the bacterial inoculum (1.5×10^8 cells). Explants remained in the inoculating dish for 1 min after the application and then returned to dosing chambers. Control explants were dosed as above except 20 μL of sterile seawater was applied in place of the bacterial inoculum.

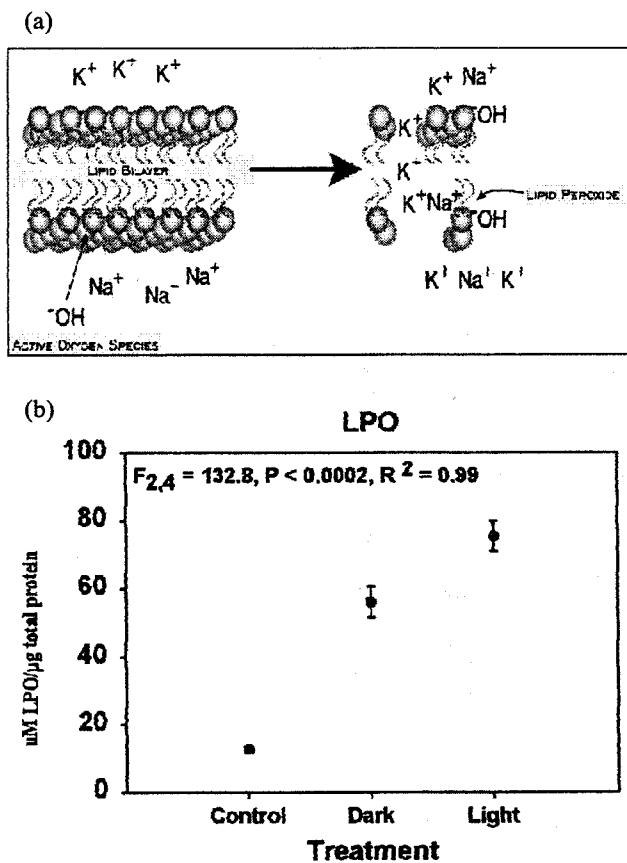


Fig 1 Lipid Peroxide (LPO). (a) Schematic of the effect of lipid peroxidation on cell membranes. (b) Mean (± 1 SE) LPO levels ($\mu\text{M}/\mu\text{g}$ total protein) in corals raised under optimal growing conditions (control), or heat-stressed in either the dark or light.

Sample Analysis

Reagents were obtained and samples were prepared for MDS analyses as reported in Downs et al. (2000, 2001). Laboratory challenge samples were assayed for at least 12 MDS parameters (Downs et al. 2000, 2001).

Results

Heat stress experiment

Treatments significantly affected all biomarkers (Downs et al. 2000). Two parameters, lipid peroxide (Fig. 1) and ubiquitin (Fig. 2) reflected the cumulative responses of the coral and its symbiont. Levels of LPO and ubiquitin differed significantly among all three treatments. Mean production of LPO was 4.4 and 5.9 times greater when corals were heat-stressed in the dark and light, respectively, than in controls. Similarly, ubiquitin accumulation was 3.3 and 4.7 times greater when corals were heat-stressed in the

dark and light, respectively, than in controls. Another parameter, the chloroplast small heat shock protein (chlpsHsp), reflected only the symbiont's physiological response to stress (Fig. 3). Levels of chlpsHsp were significantly higher in corals exposed to both heat stress and PAR than in either control or heat stress/no light treatments.

peroxidation. In contrast, protein carbonyl which reflects concentrations of protein oxidation products (Robinson et al. 1999) was lower in the bleached sample than in the uninoculated colony (Fig. 4c).

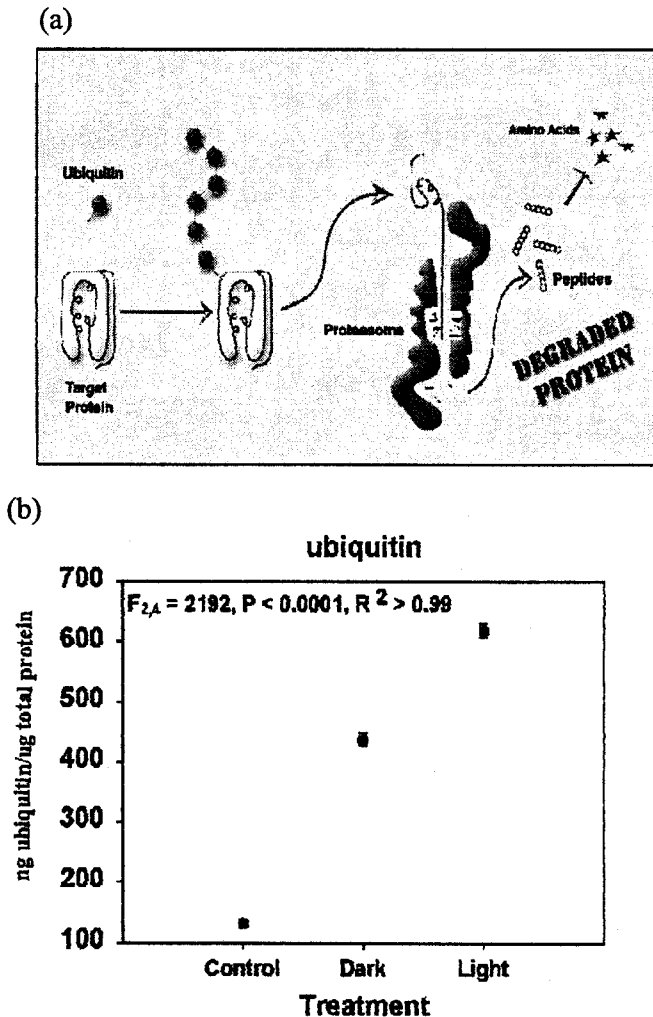


Fig. 2 Ubiquitin. (a) Schematic of ubiquitin pathway used to mark damaged proteins for degradation. (b) Mean (± 1 SE) levels of ubiquitin (ng/ug total protein) in coral explants raised under optimal conditions (control), or heat-stressed in the dark or light.

Bacterial infection

Levels of two biomarkers (malondialdehyde [MDA] and 4-hydroxy-2-trans-nonenol [HNE]) did not differ in an uninoculated colony versus one with *V. shiloi*-induced bleaching (Fig. 4a,b). These biomarkers reflect lipid

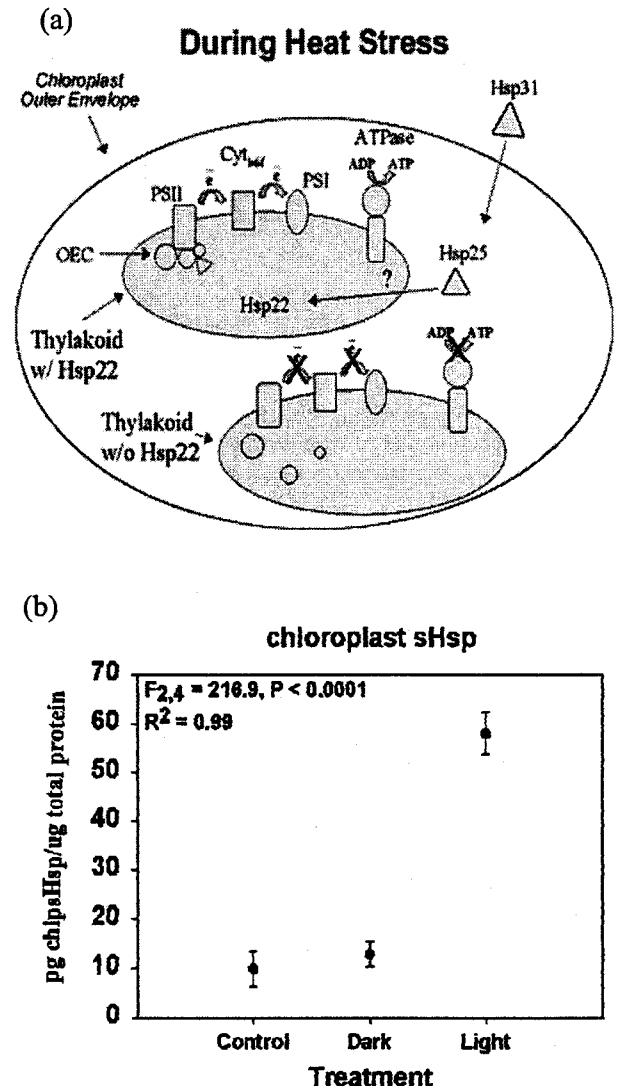


Fig. 3 Chloroplast small heat shock protein (chlpsHsp). (a) Schematic of the induction and function of the chlpsHsp during heat stress (adapted from Heckathorn et al. 1999) (b) Mean (± 1 SE) levels of chlpsHsp (pg/ug total protein) in coral explants raised under optimal conditions (control), or heat-stressed in the dark or light.

Discussion

In the laboratory, MDS parameters indicated significant differences in the physiological status of *M. faveolata* heat-stressed in the dark versus those heat-stressed in the light. Levels of cellular parameters indicative of oxidative stress

were greater when corals were heat-stressed in the light compared to corals heat-stressed in the dark, suggesting active oxygen species were generated in a light-dependent manner. The most likely source is Photosystem II (Havaux 1992; Lesser 1996, 1997; Warner et al. 1996, 1999; Downs et al., 2000) and Photosystem I (Mehler 1951). Photosynthetic-mediated generation of active oxygen species may be one cause of coral bleaching. This hypothesis suggests that bleaching occurs when active oxygen species diffuse out of the chloroplast, through the dinoflagellate, and into the coral cell, thus producing an oxidative stress within the coral itself (Lesser 1997; Brown 1997a; Warner et al. 1999). Above some threshold level, the coral then either expels or destroys the zooxanthellae as a defense against oxidative stress. Our data support this hypothesis. For example, LPO levels were significantly higher in samples heat-stressed in the light than in the dark, indicating cell membranes were damaged (Duthie 1993; Girotti 1998) and suggesting anti-oxidant defenses were also overwhelmed. Ubiquitin levels also were significantly higher in corals heat-stressed in the light than in the dark. Ubiquitin marks proteins for degradation and is an indicator of protein turnover (Goff et al. 1988; Hershko and Ciechanover 1998; Iwai 1999). These two parameters alone suggest a major insult occurred to cell structural integrity during heat stress, and was exacerbated under light conditions. Chloroplast sHsp levels were significantly higher in samples heat-stressed in the light compared both to controls, and to coral samples heat-stressed in the dark. Photosystem II is stress-labile, producing damaging active oxygen species when the oxygen-evolving complex is disrupted. The function of chlpsHsp is to protect Photosystem II activity during oxidative stress (Downs et al. 1999a; 1999b). Our data suggests bleaching occurs when chlorophyll small heat shock proteins and anti-oxidant defenses are overwhelmed.

Laboratory data for *M. faveolata* quantified molecular biomarker levels (Downs et al. 2000) under both stressful and optimal growing conditions. Preliminary testing of the MDS under natural conditions in a sibling species (*M. annularis*) sampled throughout a bleaching event (unpublished data) indicates specific diagnostic marker levels are correlated with elevated water temperatures, disruption of homeostatic mechanisms and bleaching. Although our study and others provide evidence for heat-induced coral bleaching, not all bleaching is correlated with abnormal sea surface temperatures (Oliver 1985; Fisk and Done 1985). Kushmaro et al. (1996; 1998) and Rosenberg et al. (1999) reported that bleaching of the Mediterranean coral, *O. patagonic*, resulted from bacterial infection. The causative agent (*V. shiloi*) was obtained in pure culture and caused bleaching in controlled laboratory experiments. Furthermore, infection and subsequent bleaching were temperature dependent (Kushmaro et al. 1998). We examined the cellular physiology of the temperate coral, *O.*

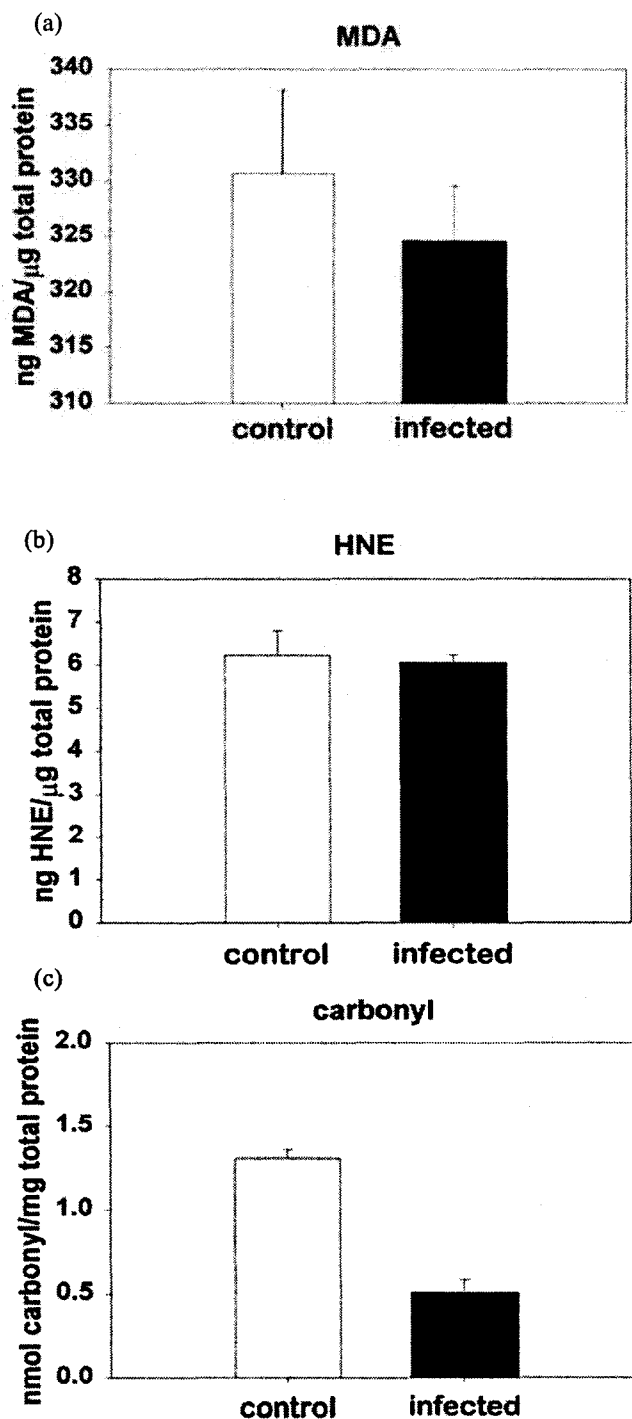


Fig. 4 Bacteria-associated coral bleaching. Biomarker levels in healthy or bacteria-induced bleached coral. (a) malondialdehyde (MDA), (b) 4-hydroxy-2-trans-nonenol (HNE), (c) protein carbonyl.

varicosa, inoculated with *V. shiloi*, using three biomarkers indicative of oxidative stress. None were elevated in a bacterial-bleached coral compared to a control, suggesting that bacterial-induced coral bleaching may not occur through an oxidative stress mechanism. Investigations are underway to characterize the differences between pathogen-associated coral bleaching and environmental bleaching (i.e., heat stress).

We introduced a newly developed biochnology and showed it can provide "environmental diagnostics" to assess the physiological status of corals. We demonstrated that it allows evaluation of single and compound stressors. We also showed that pathogen-associated coral bleaching may not result from oxidative stress. Continued use and commercialization of the MDS (EMDS™) and other newly developed molecular biomarkers, will provide scientists and resource managers the means to identify, control and possibly alleviate hidden stresses before they become environmental crises.

Acknowledgments We thank Bruce N. Ames, Barbara Brown, Phil Dustan, William Fitt, Judy Halas, Pamela Hallock-Muller, Yossi Loya, Jim Porter, Laurie Richardson, and Mark Warner for discussions of this technology in coral systems. We also thank Charles Robinson for conducting carbonyl assays; and Eric Shea for artwork on Fig. 1 & 2. Corals were collected under Florida Keys National Marine Sanctuary permits FKNMS-17-97, FWCC permit 00S517, and NMFS LOA for Scientific Research Activity. Statistical analyses were supported by NSF Grant DEB9727039 and EPA Grant R825795 to J.E. Fauth. This is EnVirtue Biotechnology, Inc. manuscript RD008.

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 Original Contribution**OXIDATIVE STRESS AND SEASONAL CORAL BLEACHING**C. A. DOWNS,^{*†} JOHN E. FAUTH,[‡] JOHN C. HALAS,[§] PHILLIP DUSTAN,^{§||} JOHN BEMISS,[†] and
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(Received 9 April 2002; Revised 30 April 2002; Accepted 2 May 2002)

Abstract—During the past two decades, coral reefs have experienced extensive degradation worldwide. One etiology for this global degradation is a syndrome known as coral bleaching. Mass coral bleaching events are correlated with increased sea-surface temperatures, however, the cellular mechanism underlying this phenomenon is uncertain. To determine if oxidative stress plays a mechanistic role in the process of sea-surface temperature-related coral bleaching, we examined corals along a depth transect in the Florida Keys over a single season that was characterized by unusually high sea-surface temperatures. We observed strong positive correlations between accumulation of oxidative damage products and bleaching in corals over a year of sampling. High levels of antioxidant enzymes and small heat-shock proteins were negatively correlated with levels of oxidative damage products. Corals that experienced oxidative stress had higher chaperonin levels and protein turnover activity. Our results indicate that coral bleaching is tightly coupled to the antioxidant and cellular stress capacity of the symbiotic coral, supporting the mechanistic model that coral bleaching (zooxanthellae loss) may be a final strategy to defend corals from oxidative stress. © 2002 Elsevier Science Inc.

Keywords—Coral bleaching, Coral reef, Oxidative stress, Oxidative damage, Stress protein response, Free radicals

INTRODUCTION

Coral reefs constitute some of the largest and most diverse ecological communities on earth and result from interactions between symbiotic organisms composed of photosynthetic dinoflagellate algae and cnidarian corals [1,2]. Over the past 30 years, coral reefs have experienced extensive degradation worldwide [2]. One etiology for this global degradation is a syndrome known as coral bleaching, a process whereby corals lose their algal symbiont or the symbiont's photosynthetic pigments degrade [3,4]. Coral bleaching can be induced by diverse factors, including high temperature, cold temperature, supersaturating light, and bacterial infection. Few models of coral bleaching that incorporate cellular and molecular mechanisms have been proposed or even tested [2–4].

Many environmental factors associated with mass

bleaching events can concomitantly induce increased production of reactive oxygen species (ROS), an antioxidant response, and significant oxidative damage to symbiotic coral [3–5]. Early studies conducted in symbiotic anemone (*Anthopleura elegantissima*) demonstrated an increased antioxidant enzymatic capacity in response to hyperbaric oxygen conditions as a result of increased oxygen production from its endosymbiotic algae [6]. Additional experiments led several workers to conclude that an increase in ROS production in *A. elegantissima* was mainly derived from light-dependent processes, such as photosynthesis [7]. Recent work from our laboratory demonstrated that there is a significant increase in oxidative damage products in coral as a result of a high-temperature and light interaction, further corroborating the conclusions of several groups [5,7–9].

One proposed model concerning a possible mechanism of coral bleaching is based on the responses to oxidative stress by both components of the symbiotic relationship [5]. Stressors such as heat stress, osmolarity, high light, and ultraviolet radiation can destabilize the

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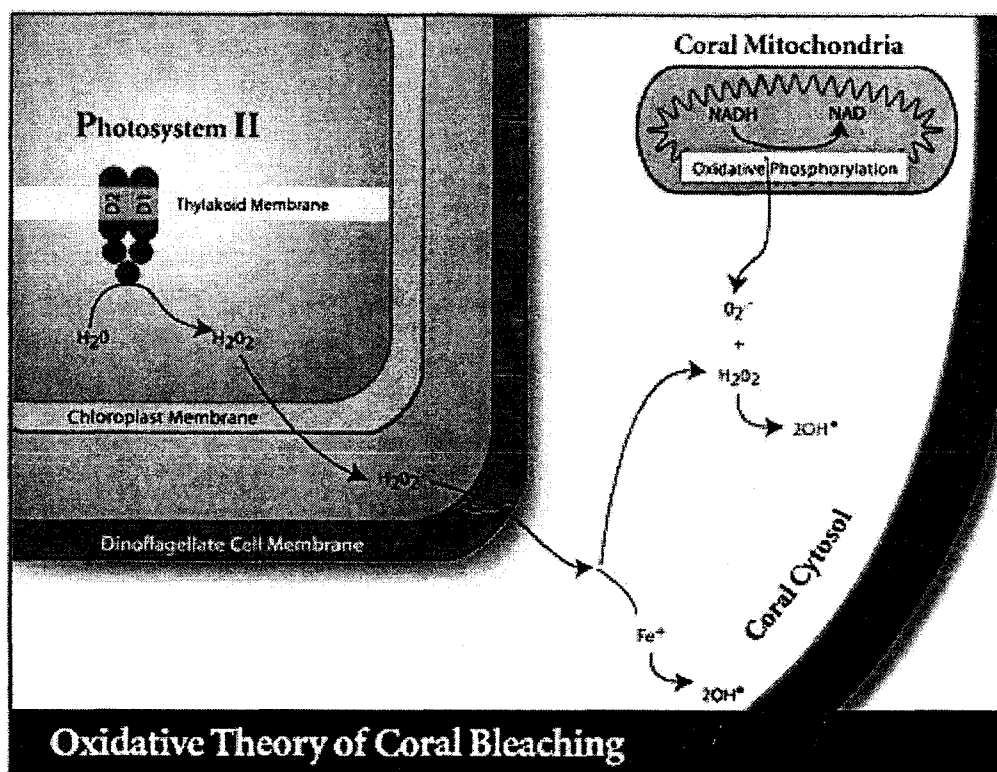


Fig. 1. Schematic representation of the proposed model in corals for the production of reactive oxygen species, their diffusion from one cellular location to another, and some of the predominant products from hydrogen peroxide as a result of increased sea-surface temperatures.

photosynthetic electron-transport chain resulting in increased production rates of reactive oxygen species (ROS) [10–13]. Production of ROS occurs in the chloroplast by several mechanisms associated with Photosystem I- and Photosystem II-catalyzed electron transfer, the most notable being the Mehler reaction and generation of hydrogen peroxide (H_2O_2) by the Oxygen-Evolving Complex [12–15]. It is proposed that algal-generated H_2O_2 can diffuse from the algal symbiont into the coral cytoplasm (Fig. 1). Once inside the coral cytosol, H_2O_2 can be either 'neutralized' by enzymatic and nonenzymatic antioxidant pathways or it can be converted into a more noxious ROS, the hydroxyl radical, either by Fenton or Haber-Weiss chemistry [10]. Below some ROS concentration threshold, the antioxidant defenses of the coral can compensate and ameliorate the destructive capacity of these ROS. Above this threshold, ROS will cause oxidative damage. At some intensity of oxidative damage, corals will eradicate the dominant source of ROS production by expelling their endosymbiotic algae. Thus, the *Oxidative Theory of Coral Bleaching* proposes that bleaching is a coral's final defense against oxidative stress (Fig. 1).

Observed correlations between global bleaching events and increased sea-surface temperatures (SST)

suggest that high temperatures may trigger oxidative-stress-driven bleaching in coral reef systems [5–9,16]. However, it is unknown if increased SST events cause coral bleaching by an oxidative-stress-induced mechanism. The primary objective of this study was to determine if levels of oxidative damage products, antioxidant enzymes, and specific components of cellular structural integrity in the star coral (*Montastraea annularis* species complex) were correlated with coral bleaching, seasonal and increased SST, and water depth in order to test the validity of certain aspects of this theory.

MATERIALS AND METHODS

Materials

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane was obtained from Millipore Corp. (Bedford, MA, USA). Dot blot and gel electrophoresis equipment were obtained from Bio-Rad Corp. (San Diego, CA, USA). GSH-420 glutathione (Cat. No. 21023) and LPO-560-assay (Cat. No. 21023) kits and cnidarian Mn superoxide dismutase (Cat. No. 24327) were obtained from Oxis International, Inc. (Portland, OR, USA). Antibodies

to α B-crystallin (Cat. No. SPA224), cnidarian heat-shock protein (Hsp)70 (Cat. No. SPA-812), cnidarian Hsp60 (Cat. No. SPA-805), cnidarian Cu/Zn superoxide dismutase (Cat. No. SOD100) were obtained from Stressgen Biotechnologies (Victoria, BC, Canada). Antibodies against algal mitochondrial small heat-shock protein (Cat. No. AB-H106), chloroplast small heat-shock protein (AB-H104), and protein carbonyl (Cat. No. X1) were obtained from EnVirtue Biotechnologies, Inc. (Harrisonburg, VA, USA). Antibody to ubiquitin and protein standards of Hsp70, Hsp60, α B-crystallin, Cu/ZnSOD, MnSOD, and ubiquitin were obtained from Sigma-Aldrich. Protein standards for the chloroplast and mitochondrial small heat-shock proteins (sHsps) were obtained from Envirtue Biotechnologies, Inc. Secondary anti-rabbit and anti-mouse alkaline phosphatase conjugated antibodies were obtained from Promega (Madison, WI, USA).

Coral sampling

Sampling was conducted within the Florida Keys National Marine Sanctuary, Key Largo, FL, USA, near Molasses Reef. This site was chosen because it exhibited a putative bleaching gradient with depth. Coral samples (<2.5 cm fragments) from five separate colonies were collected at each of four depths (ca. 3 m, 6 m, 9 m, and 18 m) with each colony sampled monthly, March–October 1999. Samples were kept in the dark by wrapping them in aluminum foil and placed in whirl-pak bags underwater. On the deck, water was removed from bags and samples immediately transferred to a liquid nitrogen dry shipper. Samples were stored at -80°C until analysis.

Assays

Each sample consisted of about eight polyps. Samples of frozen coral were ground frozen in a mortar and pestle with liquid nitrogen and weighed. Approximately 1 g of ground, frozen tissue was suspended in acetone for chlorophyll analysis as described in [17]. Roughly 4 g of ground, frozen tissue was suspended in a solution of 10 mM phosphate buffer (pH 7.8), 5 mM butylhydroxyltoluene, and 0.5% sodium dodecyl sulfate. Samples on ice were sonicated until coral tissue was homogenized. Samples were then centrifuged, and 200 μl of supernatant was aliquoted into two new tubes and stored at -80°C until sample analysis. One tube of supernatant was analyzed for protein concentration, LPO and total GSH content as previously described [5]. To the other tube of supernatant, a 20 μl solution containing 20% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol,

100 mM ethylenediaminetetraacetic acid, 10 mM sorbitol, 15% polyvinylpyrrolidone (wt/vol), 3% polyvinylpyrrolidone (wt/vol), 20 mM phenylmethylsulfonyl fluoride, 20 mM benzamide, 50 μM α -aminocaproic acid, and 1 $\mu\text{g}/100$ μl pepstatin A was added to the sample tube. Samples were boiled for 3 min, allowed to sit at 25°C for 5 min, and then centrifuged at $10,000 \times g$ for 5 min. Supernatant absent of a lipid/glycoprotein mucilage matrix was transferred to a new tube and subjected to another protein concentration assay [18]. Samples were then assayed by ELISA for α B-crystallin homologue, chloroplast small heat-shock protein, cnidarian Hsp70, cnidarian Hsp60, cnidarian Cu/Zn SOD, cnidarian MnSOD, total algal small heat-shock protein content, and total ubiquitin as described in [5]. Because of the extensive protein glycosylation and polysaccharide content found in corals, protein carbonyl content was assayed using a modified method of Robinson et al., which takes into account carbonyl formation as a result of technique artifact and nonprotein carbonyls, such as those found in DNA, RNA, and polysaccharides [19].

Statistical analysis

We used repeated measures multivariate analysis of variance (MANOVA) to test whether mean biomarker levels differed over time, among depths, and with the time $\times$ depth interaction. We used univariate tests to interpret significant multivariate responses, and the Tukey-Kramer Honestly Significant Difference (HSD) method to compare univariate means. Levels of GSH, LPO, and Hsp 70 were log-transformed to meet analysis assumptions. To avoid temporal pseudoreplication, we used effective sample sizes (n_e) to determine the significance of correlations between time series [20]. Effective sample sizes adjust for first-order autocorrelations; if either series has zero first-order autocorrelation, no reduction is required in the original sample size. All statistical analysis were conducted using JMP V. 3.2 (SAS Institute, Inc., Cary, NC, USA) or higher, with $\alpha = 0.05$ for all tests.

RESULTS AND DISCUSSION

Oxidative stress is suggested to play a role in coral bleaching, though substantial evidence is lacking for a relationship between coral bleaching, oxidative stress, and environmental factors that are relevant to phenomena occurring in the field. More importantly, there is an absence of cellular-based mechanistic models concerning oxidative stress and coral bleaching that could be tested in both the laboratory and in the field. Based on early work in anemones and laboratory work on corals,

we propose a model of oxidative-stress driven coral bleaching [5]. High-temperature destabilization of Photosystem II occurs, in part, by the impairment of the oxygen-evolving complex (OEC) [21–23]. One mechanism of OEC impairment is associated with the release of Mn^{2+} and Cl^- from OEC and the dissociation of proteins OEC18, OEC24, and Photosystem II-associated heme catalase [24–27]. As a result, the rate of hydrogen peroxide generation increases by orders of magnitude [25]. Heat stress can further exacerbate the potential for hydrogen peroxide-mediated oxidative stress through the deactivation of a number of hydrogen peroxide-neutralizing pathways, allowing for concentrations to increase [28–30]. Diffusion of hydrogen peroxide out of the chloroplast and into other dinoflagellate organelles and the cnidarian cytoplasm can result in oxidative damage through the entire symbiotic organism, especially in organelles that contain high iron content or are associated with production of superoxide (e.g., mitochondria) [10]. In response to this oxidative stress, the coral expels the dinoflagellate, resulting in the phenomenon observed as coral bleaching.

This model for the oxidative theory of coral bleaching also posits that variation of the occurrence of bleaching should be related to specific molecular mechanisms that prevent the production of ROS or protect against the damaging effects of ROS. For example, increase in the levels of Cu/Zn SOD should be associated with an increase in the threshold at which oxidative damage occurs, because of the reduction of available substrates for Haber-Weiss chemistry [31]. Other protective mechanisms include antioxidant pathways, such as the Asada-Halliwell pathway and xanthophylls cycling, as well as 'preventive' mechanisms such as the production of small heat-shock proteins, changes in lipid composition, and production of stress-stable components of labile enzymatic complexes of pathways associated with electron transport [10,32–34].

The validity of a mechanistic model should be reflected in the accuracy at which it can predict the occurrence of phenomena at various hierarchical levels [35]. For example, if oxidative damage is an initiating event in the expulsion of a coral's zooxanthallae (its algal symbiont), a punctuated increase in levels of oxidative damage products should be detected before bleaching is observed. Furthermore, higher levels of antioxidant components (e.g., ascorbate peroxidase, glutathione peroxidase) in one individual should be associated with a different threshold or occurrence for bleaching compared to an individual that has lower levels of these antioxidant components [10]. This analysis can also help determine the relative contribution and role of different antioxidant and protective pathways (e.g., heat-shock protein response) in the process of coral bleaching [36].

Sea temperatures, at depth, beside each group of five coral heads, recorded at the time of sample collection, varied significantly among months (Fig. 2a). Temperatures at all sites exceeded 28°C from June–September, and peaked in July, when temperatures at the two deepest sites reached or exceeded 30°C. By September, all colonies at the 18 m site exhibited extensive bleaching, and colonies at the 9 m site either were partially bleached or had moderate paling. In contrast, corals at the 3 m site did not exhibit any visual symptoms of bleaching (Fig. 2b). The bleached or nonbleached appearance of colonies was related to July ocean temperature (logistic regression, $\chi^2 = 21.0$, $p < .0001$; $r^2 = 0.76$). The extent of bleaching was quantified using chlorophyll *a* concentration, which was negatively correlated with ocean temperature ($r = -0.53$, $n_e = 23.6$, $p < .01$). Temperatures at all sites remained above 28°C until October, when water temperature decreased over a 2 week period to less than 25°C in the 3–9 m sites, and below 27°C at the 18 m site. By October, colonies at all depths darkened in color and increased in chlorophyll *a* content indicating recovery from bleaching (Fig. 2c). Hence, coral bleaching was not a universal occurrence at high sea-surface temperatures, but was positively associated with depth.

Oxidative stress is defined as an imbalance in the pro-oxidant/antioxidant ratio, which favors increased pro-oxidants and results in oxidative damage [10]. Previous work from our laboratories demonstrated that heat stress causes oxidative damage in corals and that damage is exacerbated by exposure to light [5]. To test whether oxidative damage is associated with and precedes coral bleaching we examined levels of protein carbonyl and lipid peroxidation (LPO), both of which reflect aspects of cellular oxidative damage. Mean carbonyl concentration differed significantly with the exact combination of sampling date and depth (Fig. 3a), and was positively correlated with ocean temperature ($r = +0.47$, $n_e = 23.9$, $p < .05$). Lipid peroxide (LPO) showed a similar pattern (Fig. 3b). As water temperatures increased, so did levels of oxidative damage products. A specific pattern emerged: corals at the greater depths had higher levels of oxidative damage, especially protein carbonyl. There was a statistically significant negative correlation between carbonyl and chlorophyll *a* content ($r = -0.22$, $n_e = 102.0$, $p < .01$), indicating that increased oxidative damage levels were associated with bleaching. Furthermore, oxidative damage products increased as water temperature increased and preceded coral bleaching.

Cellular damage, such as that induced by oxidative stress, often increases protein chaperone and protein turnover activity [5,10,37,38]. To determine if increasing oxidative damage products were associated with decreasing scleractinian cellular integrity (i.e., to determine if corals were experiencing an oxidative stress), we exam-

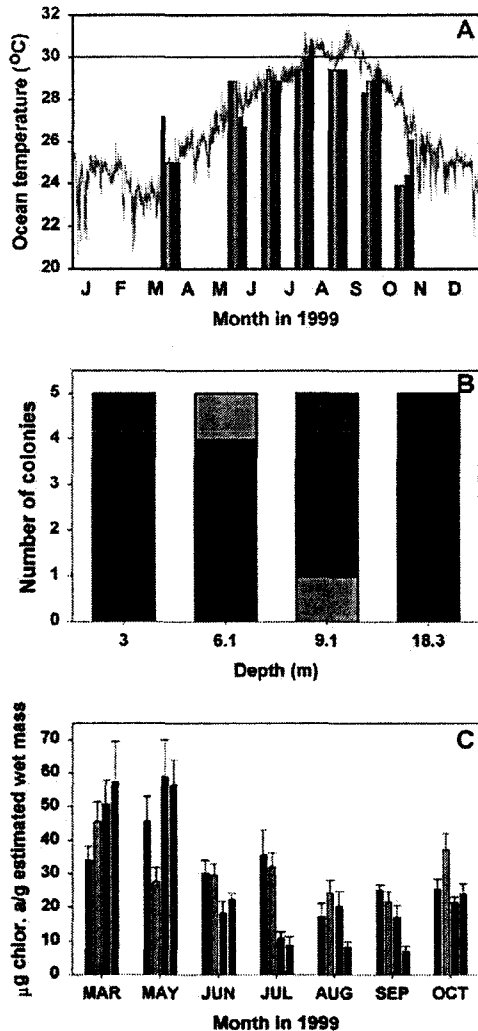


Fig. 2. (A) Seasonal variation in ocean temperatures recorded beside coral (*Montastraea annularis*) colonies at four depths. Bars show depth of colonies: black = 3 m, gray = 6 m, red = 9 m, blue = 18 m. Temperature in summer months (June–September) differed significantly from all others except May [two-way ANOVA: month effect: $F(6,18) = 23.3$, $p < .0001$; depth effect $F(3,18) = 0.19$, $p > 0.90$; Model $R^2 = 0.89$]. Yellow line is data from the C-Man Buoy at Molasses Reef, within 1–3 miles from sampling sites. Green line indicates the temperature threshold at which bleaching often is observed (1–4, 6, 7). (B) Colony condition in September, 1999 varied significantly among depths (log likelihood ratio test, $G = 27.95$, 6 df, $p < .0001$; $r^2 = 0.74$). Healthy colonies, black bars; paling colonies, gray bars; bleached colonies, red bars. (C) Chlorophyll *a* levels reflect photosynthetic activity and density of symbiotic zooxanthellae inhabiting coral tissue, providing a quantitative measure of bleaching. Bars show mean (± 1 SE) concentration of chlorophyll *a* in five coral colonies repeatedly sampled at each depth: black = 3 m, gray = 6 m, red = 9 m, blue = 18 m. Differences among months and the month $\times$ depth interaction were significant [repeated measures MANOVA: month effect $F(6,11) = 14.68$, $p < .0001$; month $\times$ depth interaction, $F(18,31.6) = 2.53$, $p < .011$].

ined levels of scleractinian Hsp60, scleractinian Hsp70, and ubiquitin. Chaperonins Hsp60 and Hsp70 increased

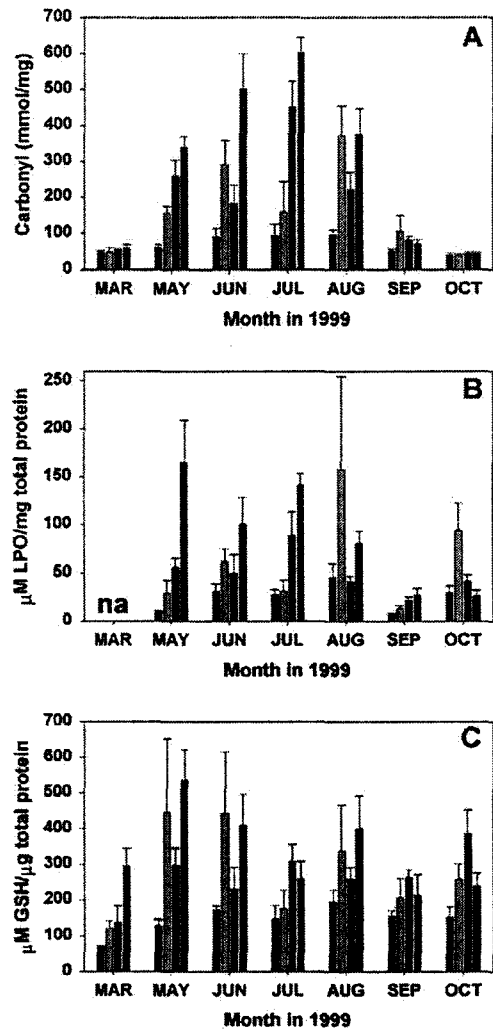


Fig. 3. Oxidative damage. (A) Protein carbonyl concentrations reflect protein oxidative damage, which varied significantly with depth, month, and the depth $\times$ month interaction (repeated measures MANOVA: all $F > 3.96$, $p < .0004$). (B) Lipid peroxidation reflects damage to cell and organelle membranes, and $\log_{10}$ -transformed means varied significantly with depth, month, and the depth $\times$ month interaction (repeated measures MANOVA: all $F > 2.47$, $p < .015$). Lipid peroxide was not assayed (na) in March. (C) GSH reflects cell redox status and is an antioxidant; its $\log_{10}$ -transformed means varied significantly with depth, month, and the depth $\times$ month interaction (repeated measures MANOVA: all $F > 2.54$, $p < .035$). Bars show untransformed mean (± 1 SE) biomarker concentrations at each depth: black = 3 m, gray = 6 m, red = 9 m, blue = 18 m.

significantly at all sites in May compared to March (ANOVA, both $F > 32.1$, $p < .0001$; Figs. 5A and 5B). From May–September, Hsp60 levels were significantly lower in corals at the 3 m site compared to corals at all other sites, except the 6 m site in July–August, which was equivalent [one-way ANOVA followed by Tukey–Kramer HSD Test: all $F(3,16) < 5.93$, $p < .01$]. Levels of Hsp70 showed a similar pattern from March–July,

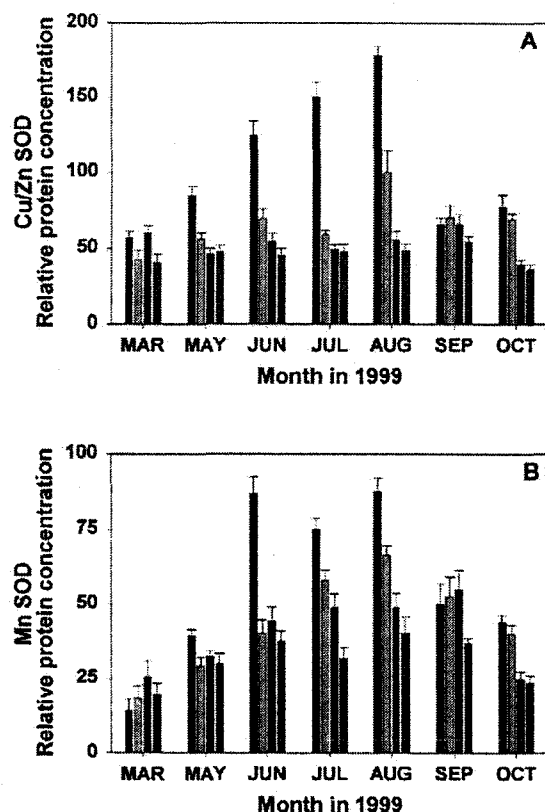


Fig. 4. Levels of cnidarian superoxide dismutases. (A) Cu/Zn superoxide dismutase protein levels reflects overall cnidarian responses, which varied significantly with depth, month, and the depth $\times$ month interaction (repeated measures MANOVA: all $F > 6.84$, $p < .0001$). (B) Mn superoxide dismutase protein levels reflects only responses of cnidarian mitochondria, which varied significantly with depth, month, and the depth $\times$ month interaction (repeated measures MANOVA: all $F > 6.95$, $p < .0001$). Symbols are as in Fig. 3.

indicating increased chaperone activity during these months (Fig. 5B). However, elevated chaperone levels do not always indicate cellular damage [39]. To differentiate between increased chaperone activities resulting from cellular damage versus cellular growth, we examined changes in ubiquitin levels [37]. In May, ubiquitin levels were 2.5 to 5.0 times higher than in March [Welch ANOVA, $F(1,20.3)$, $p < .0001$; Fig. 4C]. Furthermore, there was a positive correlation between ubiquitin and protein carbonyl levels ($r = +0.61$, $n_e = 122.5$, $p < .0001$), indicating increased ubiquitin production and accumulation was associated with increased protein damage. Positive correlations between Hsp70 and carbonyl content during the whole sampling season ($r = 0.62$, $n_e = 96.1$, $p < .01$), and especially from June through September ($r = 0.55$, $n_e = 48.1$, $p < .01$) corroborated this interpretation. Further corroboration for this interpretation was found with positive correlations between Hsp60 and carbonyl content, both during the whole sampling season ($r = 0.55$, $n_e = 70.6$, $p < .0001$).

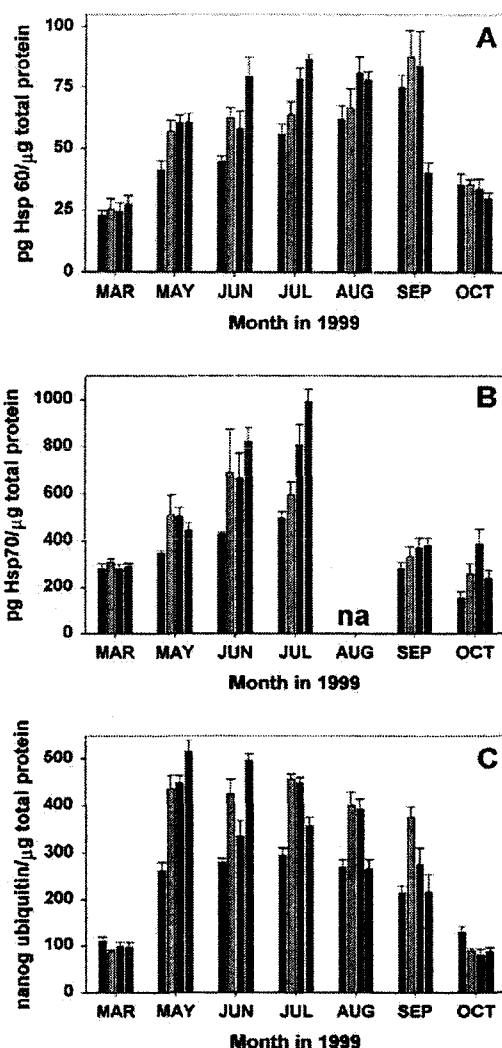


Fig. 5. Levels of chaperonins. (A) Hsp60 and (B) Hsp70 reflect chaperonin levels in the cnidarian and not of the dinoflagellate; mean concentrations of both varied significantly with depth, month, and the depth $\times$ month interaction (repeated measures MANOVAs: all $F > 2.56$, $p < .02$; Hsp70 $\log_{10}$ -transformed prior to analysis). Hsp70 was not assayed (na) in August. (C) Ubiquitin levels reflect the rate of protein degradation, which varied significantly with depth, month, and the depth $\times$ month interaction (repeated measures MANOVA: all $F > 8.80$, $p < .0001$). Symbols are as in Fig. 3.

.01) and between June and September ($r = 0.35$, $n_e = 70.6$, $p < .01$).

Total glutathione content could be used, in part, as an index of antioxidant capacity of an organism. Changes in glutathione concentration could also be used as a proxy for a response to specific stressors (e.g., electrophilic xenobiotic or ROS) [5,10,40]. For example, increases in glutathione are observed after a stress event and have been suggested as a means of physiologically acclimating to the stressor [10,40]. We measured total glutathione

concentrations to determine if corals increased their antioxidant capacity either in response to sea-surface temperatures or oxidative damage products. Mean GSH content differed significantly with the exact combination of sampling date and depth (Fig. 3C), with corals at the 3 m site always having lower mean GSH concentrations than those at deeper sites. Mean GSH content was independent of ocean temperature ($r = +0.16$, $n_e = 25.4$, $p > .25$), but significantly correlated with accumulations of the oxidative damage products carbonyl and LPO ($r = +0.42$ and $+0.49$, respectively; both $n_e > 109$ and $p < .0001$). One interpretation of the GSH analyses is that a coral will increase its antioxidant capacity in response to increasing oxidative damage.

To corroborate this interpretation, we examined other aspects of coral antioxidant status, including copper/zinc (Cu/Zn) and manganese (Mn) superoxide dismutase accumulation (SOD). Both SOD proteins reflected only the cnidarian response, not those of the dinoflagellate zooxanthellae [5]. Copper/Zinc SOD concentrations varied significantly with the exact combination of sampling date and depth (Fig. 4A). From May–August, Cu/Zn SOD concentrations were significantly higher at the 3 m site than at all deeper sites [one-way ANOVA followed by Tukey-Kramer HSD Test: all $F(3,16) < 14.9$, $p < 0.0001$]. Manganese SOD, an enzyme found only in mitochondria, showed a similar accumulation pattern (Fig. 4b). Levels of Mn SOD and Cu/Zn SOD were significantly positively correlated ($r = 0.75$, $n_e = 77.6$, $p < .0001$). From March to October, mean accumulations of Mn SOD and Cu/Zn SOD were negatively correlated with *variances* in carbonyl ($-0.70 \leq r \leq -0.52$; all $p < .02$, $n = 20$). Furthermore, between June and September levels of both Cu/Zn SOD and Mn SOD were positively correlated with chlorophyll *a* content ($r = 0.33$, $n_e = 50.9$, $p \leq .05$; $r = 0.38$, $n_e = 97.2$, $p \leq .05$, respectively). Both of these analyses strongly suggest that superoxide dismutases are significant components of the antioxidant capacity of corals and that these two proteins may play an important role in preventing the process of coral bleaching.

sHsps protect various metabolic pathways and cellular functions from heat and oxidative damage [32]. Specific sHsps have been demonstrated to protect glycolytic and electron-transport enzymes against oxidative damage, such as those in photosynthesis and respiration [41–43]. Measurement of specific sHsps can help determine whether (i) specific metabolic processes are being stressed, and (ii) gauge the capacity to which these metabolic processes can endure a stress [5,32,40]. We examined three species of sHsps in the symbiotic coral: chloroplast sHsp of the dinoflagellate, dinoflagellate mitochondrial sHsp, and t scleractinian α B-crystallin immunohomologue.

The chloroplast sHsp is evolutionarily conserved and usually is only present in response to a stressed condition [44]. This protein specifically associates with the oxygen-evolving complex of photosystem II and protects this enzyme's function during heat stress, high-light stress, ultraviolet-light exposure, and oxidative stress, most likely via a recycling antioxidant mechanism [43, 45]. Measurement of the chloroplast sHsp is relevant for two reasons: (i) photo-oxidative damage to photosystem II within the zooxanthellae has been implicated in heat-stress-mediated bleaching and (ii) photosystem II is a primary generator of hydrogen peroxide as a result of a degenerating efficiency of the oxygen-evolving complex in hydrolyzing water to diatomic oxygen [15,28,30,46, 47]. Chloroplast sHsps were barely detectable in corals at all depths in March, but increased dramatically in May [Welch ANOVA, $F(1,19.3) = 54.6$, $p < .001$; Fig. 6A]. Levels of this protein were greatest in corals at 3 m and decreased with depth, a pattern that remained consistent throughout the year. Chloroplast sHsp levels were negatively correlated with protein carbonyl ($r = -0.55$, $n_e = 72.6$, $p < .0001$) and LPO ($r = -0.38$, $n_e = 113.4$, $p < .001$) levels during May–September, when ocean waters were warmest. Hence, during heat stress, increased chloroplast sHsp concentrations may have protected zooxanthellae by preventing increased production of ROS. More importantly, the negative correlation between accumulation of chloroplast sHsp and oxidative damage products suggests that Photosystem II may be the principal component responsible for generating the oxidative stress associated with bleaching.

In vascular plants, the mitochondrial sHsp specifically associates with and protects the NADH:ubiquinone oxidoreductase complex of oxidative phosphorylation during heat and oxidative stress [41,48]. Less is known concerning the specific function and localization of the mitochondrial sHsp in dinoflagellates, although *in vitro* experiments indicate its expression is stress inducible (C. Downs, unpublished data). Mitochondrial sHsps levels showed a pattern of expression similar to that of the chloroplast sHsp in June to August, but mitochondrial sHsp levels did not differ significantly among the 6 m, 9 m, and 18 m sites in September and October (Fig. 6B). Mitochondrial sHsp levels were negatively correlated with protein carbonyl levels during May–September ($r = -0.34$, $n_e = 46.2$, $p < .05$; Fig. 6B), again suggesting a possible protective effect with higher expression of this protein. Furthermore, this data suggests dinoflagellate mitochondria were experiencing and responding to stress, whether in response to increased SST, the ROS generated by the chloroplast, or both.

In both vertebrates and invertebrates, homologues of α B-crystallin are usually found at some basal level and hyper-accumulate in response to a stressed condition

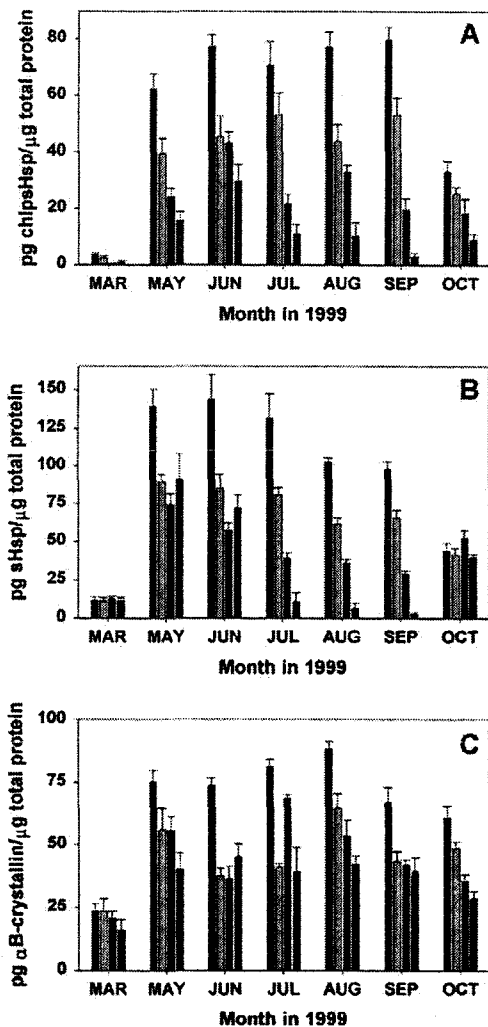


Fig. 6. Levels of small heat shock proteins. (A) Chloroplast sHsp levels varied significantly with depth, month, and the depth $\times$ month interaction (repeated measures MANOVA: all $F > 8.32$, $p < .0001$). (B) Dinoflagellate mitochondrial sHsp levels varied significantly with depth, month, and the depth $\times$ month interaction (repeated measures MANOVA: all $F > 7.28$, $p < .0001$). (C) In the cnidarian, levels of the immuno-homologue of α B-crystallin varied significantly with depth, month, and the depth $\times$ month interaction (repeated measures MANOVA: all $F > 3.46$, $p < .01$). Symbols are as in Fig. 3.

[5,49]. Evidence suggests that this protein species acts as a molecular chaperone, suppressing the unfolding and aggregation of proteins by trapping early unfolding intermediates, thereby increasing the amount of energy required to attain structural transition states [50]. Evidence from vertebrate research indicates that α B-crystallin interacts and stabilizes cytoskeletal elements during stress [51,52]. In corals, a 19 kDa immuno-homologue of this protein exists with an unknown function [5]. Accumulations of the immuno-homologue to α B-crystallin specific to the scleractinian showed a complicated pat-

tern (Fig. 6C). This protein increased one- to two-fold from March to May, and maintained these levels throughout the year. Like the other two sHsp homologues, levels of α B-crystallin were negatively correlated with carbonyl levels during May–September ($r = -0.24$, $n_e = 91.5$, $p < .025$).

In summary, the data presented here support specific aspects of the oxidative stress model of coral bleaching. We demonstrate (i) that the concentration of specific oxidative damage products were highly correlated with coral bleaching, (ii) severe oxidative damage preceded loss of chlorophyll and coral bleaching, (iii) that oxidative damage was associated with increased damage to aspects of cellular integrity, (iv) that increased energy expenditure was required to maintain homeostasis during conditions associated with bleaching, (v) components of cellular defenses (e.g., antioxidant defense and stress-protein response) played a role in protecting against bleaching, and (vi) evidence that Photosystem II is a primary source for ROS production, hence a principal element in oxidative stress and temperature-associated coral bleaching.

Although mass coral bleaching events are often associated with increased sea-surface temperatures, spatial variation in the frequency and intensity of bleaching suggests both corals and their zooxanthellae vary in their individual responses to heat and light stress [3]. For example, different species of coral have different temperature thresholds for coral bleaching [53,54]. Furthermore, there is significant variation among individuals of the same species in their threshold of bleaching. This variation is correlated with components of cellular defense against stress, indicating that these molecular/cellular processes could be the focal point for such variation. The argument that bleaching susceptibility has a genetic component is further supported by studies demonstrating strong correlations between specific genotypic clades of dinoflagellates and incidence of bleaching [55].

Our results also emphasize the importance of further investigations of both cnidarian and dinoflagellate cellular-stress physiology. For example, we only measured levels of Cu/Zn SOD and Mn SOD in the cnidarian. Concentrations and activity of these enzymes in the dinoflagellate, as well as FeSOD, need to be investigated. Oxidative defensive mechanisms that specifically protect photosynthesis, such as aspects of the Photosystem II-associated heme catalase and the xanthophyll cycle, should also be examined and characterized. Mechanisms and pathways other than those of the classical antioxidant defenses should be examined to determine if these are significant processes that mitigate coral bleaching. For example, there are several ORFs in the dinoflagellate chloroplast genome that show significant homology of the *psbA* gene of the D1 protein of photosystem II [56].

In *Synechocystis*, differential induction of the *psbA* genes are associated with tolerance against conditions that induce photoinhibition and production of ROS [36,57,58]. Preliminary evidence from a heat-stressed subtractive library of dinoflagellates obtained from the coral *Montastraea annularis* indicates differential induction of *psbA* transcript homologues, suggesting that a similar mechanism of protection may exist in coral dinoflagellates (Downs, C., Markus, E., Vetter, L., unpublished results). Other adaptations to stress, such as changes in lipid composition of key organellar membranes, induction of scleractinian sHsps, and shifts in metabolic pathways, need to be investigated so that their relative contribution and role in coral stress tolerance can be determined.

Coral bleaching does not always result in coral mortality. There are a number of observations both from the laboratory and the field documenting recovery from a bleaching event [2,3]. One hypothesis for this phenomenon, especially given the data supporting the *Oxidative Theory of Coral Bleaching*, is that the outcome for either mortality or recovery is determined by the extent of oxidative damage experienced by the coral. Irreparable damage to essential cellular structures, such as chromosomes and mitochondria, by ROS can initiate the development of apoptotic or necrotic processes [10]. Minor damage can be repaired and the coral recover, as suggested by the increase in chaperone and protein turnover activity (Figs. 3 and 4). Such a hypothesis can be easily tested, both in the laboratory and in the field, using many of the methodologies and techniques first developed in mammalian studies of ROS-toxicity and pathologies [10].

Finally, chronic or repeated exposures to conditions leading to oxidative stress in corals could have significant impacts to coral ecology that are not readily observable. Many corals, especially the reef building coral species, are long-lived (ca. 200–800 years old). Climatic/oceanic events associated with mass-coral bleachings are argued to have increased in frequency and intensity over the past 40 years in contrast to what has occurred in the last 450–6000 years [2,59]. Some of these events, especially El Niño phenomenon, result in increased sea-surface temperatures that are oftentimes at or above coral bleaching thresholds [3]. By extrapolating some of the tenets of the *Free Radical Theory of Aging* to coral biology, it can be hypothesized that chronic or repeated exposures to conditions conducive to oxidative stress may decrease the overall condition of coral [60]. For example, a decrease in the fitness of the individual or its offspring may result from a decrease in the overall genomic integrity that is usually associated with oxidative stress [10,60]. Such a decline could be further detected as an increase in mutation rate, decline in the

stress-protein response, or a decline in enzymatic activity necessary for homeostasis [61]. It has been suggested that many cnidarian species are 'immortal' or lack the deteriorative process associated with senescence [62]. In light of the findings presented here and in the literature, increased significance must be given to the question: are oxidants a determinant of the maximum life span and condition of corals, thereby influencing coral reef ecology?

Acknowledgements — We thank Judy Halas and William Fitt for discussion and support of this work; Mike Brill, Karen Bareford, Darren Wray, Claire Chandler, Hank Becker, and Amy Massey for establishing sites, sample collection, and sample preparation; and Charles E. Robinson, Arnold Huang, and Bruce N. Ames for discussion of oxidative stress, assay development and critical comments on this report. C. Downs thanks Bruce N. Ames for his encouragement during the writing of this manuscript at the University of California, Berkeley. Statistical analyses and manuscript preparation were supported by National Science Foundation grant DEB9727039 and Environment Protection Agency grant R825795 to J. E. Fauth. Corals were collected under permit FKNMS-99-042. This is Envirtue Biotechnologies, Inc. manuscript RD002.

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Mid-range Prediction of Coral Bleaching: A Molecular Diagnostic System Approach

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Abstract

One challenge facing ecological forecasters is making accurate, mid-range predictions of changes to ecosystem health. Accurately forecasting disease and degradation months in advance could allow resource managers time to minimize or ameliorate stressors that threaten ecosystem structure, function, and integrity.

*This research has resulted in a molecular biomarker system to assess the physiological status of corals by assaying specific parameters of cellular physiology indicative of individual colony health. Field samples of boulder corals (*Montastraea annularis*) from the Florida Keys, USA, showed a strong correlation between molecular biomarker levels and bleaching of individual coral colonies. Logistic regression identified molecular biomarkers capable of predicting the onset of coral bleaching 3-6 months before symptoms became apparent.*

Biomarkers useful in forecasting included glutathione (an antioxidant), lipid peroxides (which indicate functional lipid levels and membrane integrity) and chloroplast small heat shock proteins (chlpsHsp, which protect photosystem II activity during oxidative stress). Relationships observed between water depth, biomarker levels, and bleaching suggest deep-water corals cannot accumulate enough chlpsHsp to prevent Photosystem II from dissociating and generating the oxidative stress that causes bleaching.

Molecular biomarkers provide useful tools for ecological forecasting because they assay vital cellular and physiological parameters. Understanding the mechanistic basis of degradative processes should help scientists and resource managers identify and alleviate the environmental stresses that trigger them.

Introduction

Accurate ecological forecasts made months in advance can give resource managers time to minimize or ameliorate stresses that threaten ecosystems (Clark *et al.* 2001, National Biological Information Infrastructure 2001). Forecasting stress responses to intervene and prevent damage requires linking changes at lower levels of biological organization with the fitness of individuals, and accurately assessing the long-term, ecosystem-level consequences.

Stress often indirectly affects ecosystems by overwhelming defenses at lower levels of the biological hierarchy: molecular, cellular, and organism-level homeostatic processes. Stress then reduces individual fitness, alters demographic parameters, and diminishes the structure, function, and resilience of communities which comprise the biotic component of ecosystems. Changes in molecular and cellular parameters may precede ecosystem-level responses to chronic stress by days, months, or years.

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A molecular diagnostic system (MDS) has been developed to monitor cellular stress responses and to develop forecasting capabilities (Downs *et al.* 2000, Downs, Fauth & Woodley 2001, Downs, Dillon & coauthors 2001, Woodley *et al.* 2001, Downs, Shigenaka & coauthors 2002, Downs, Fauth & coauthors 2002). The MDS assays specific cellular and molecular parameters indicative of cellular physiological status, including whether 1) cell structural integrity was compromised, 2) cells experienced an oxidative stress, and 3) defenses were mounted to a particular stress (i.e., heat, pesticide, heavy metal).

Using molecular biomarkers to assess organism or ecosystem health is a popular idea with considerable evidence of its effectiveness (reviewed by Feder & Hoffman 1999). However, most studies examine only a single or several physiological parameters to gauge overall response to acute and chronic stress. Surprisingly few studies evaluate overall physiological status with multiple, specific molecular biomarkers (Schaeffer *et al.* 1988, Stegeman *et al.* 1992, Joanisse & Storey 1996a,b, Downs *et al.* 2000, Downs, Fauth & Woodley 2001, Downs, Dillon & coauthors 2001, Woodley *et al.* 2001, Downs, Shigenaka & coauthors 2002, Downs, Fauth & coauthors 2002). The goal is to develop a comprehensive molecular diagnostic system to assess ecosystem health, providing resource managers timely information to better understand, monitor, and manage at-risk ecosystems.

While in theory the MDS can be applied to any living organism, the first forecast to be developed was for bleaching of reef-building corals in the Florida Keys National Marine Sanctuary (FKNMS), USA. The forecast arose from a research partnership between the Center for Coastal Environmental Health and Biomolecular Research (US National Oceanic and Atmospheric Administration/National Ocean Service), the FKNMS, EnVirtue Biotechnologies, Inc., and academic scientists. There were three objectives: 1) to identify probable mechanisms of coral bleaching, 2) to identify factors that influenced susceptibility of individual coral colonies to bleaching, and 3) to develop a method of forecasting bleaching of individual coral colonies. Once tested and verified, forecasts could be used by National Marine Sanctuary managers to identify vulnerable coral reefs, to assess the relative importance of different stresses on coral bleaching, and to set quantitative recovery endpoints for restoring reefs degraded by chronic stress.

Case Study

During bleaching, corals lose their symbiotic algae and/or their photosynthetic pigments. Bleaching robs corals of nutrients, slows growth (Hughes and Tanner 2000) and recovery from injuries (Mascarelli and Bunkley-Williams 1999), increases susceptibility to diseases (Kushmaro *et al.* 1996), delays reproduction (Hughes and Tanner 2000, Omori *et al.* 2001), and can kill entire colonies (Carriquiry *et al.* 2001).

Bleaching is caused by diverse stresses (reviewed in Williams and Bunkley-Williams 1990, Brown 1997a,b). However, heat stress is the primary trigger of mass bleaching events that have degraded coral reefs worldwide over the past 20 years (Williams and Bunkley-Williams 1990, Goreau *et al.* 1992, Goreau and Hayes 1993, Brown *et al.* 1996, Brown 1997a,b). Recently, Stone and co-workers (Huppert and Stone 1998, Stone *et al.* 1999) showed episodes of mass coral bleaching were associated with increased severity and frequency of El Niño events, which are symptomatic of global warming. Increased ocean temperatures compounded with other agents of coral degradation (e.g., pollution, disease, predation) pose an increasing and significant threat to the health and vitality of coral reef ecosystems worldwide (Dustan 1999).

The US National Oceanic and Atmospheric Administration currently uses remote sensing of unusually warm surface waters to identify areas where bleaching may occur (Carriquiry *et al.* 2001, NOAA National Environmental Satellite, Data, and Information Service 2002). However, even during extreme sea surface temperature anomalies, phylogenetic, spatial, temporal, bathymetric, and size-related variation in coral bleaching and mortality (Williams and Bunkley-Williams 1990, Hughes and Tanner 2000, Hueerkamp *et al.* 2001, Loya *et al.* 2001) can obscure both the intensity of bleaching events and their underlying causes. This is the situation for corals along the Florida Keys, which are subjected to different stressors from Key Largo to the Dry Tortugas (Dustan 1999, Porter *et al.* 1999).

A series of recent papers (Downs *et al.* 2000, Woodley *et al.* 2001, Downs, Fauth & coauthors 2002) demonstrated how heat stress affects coral MDS parameters under both laboratory and field conditions. In the laboratory, corals (*Montastrea faveolata*) heat-stressed under normal photosynthetically active radiation (PAR) produced more chloroplast small heat shock

protein (chlpHsp) than either the controls or corals heat-stressed in the dark (Downs *et al.* 2000, Woodley *et al.* 2001). Chloroplast small heat shock protein protects Photosystem II activity during oxidative stress (Downs *et al.* 2000, Downs, Fauth & coauthors 2002); when this defense is overwhelmed, oxyradicals are produced. In cnidarians, these reactive oxygen species cause lipid peroxidation, protein degradation, and DNA damage, and ultimately tissue and cell death (Hawkrige *et al.* 2000).

As expected, corals heat-stressed under PAR had significantly higher levels of lipid peroxide than those heat-stressed in the dark, indicating greater damage to lipid membranes. A similar pattern was seen in ubiquitin levels, which signals protein degradation (Downs *et al.* 2000). Superoxide dismutases (SOD) such as Cu/Zn and Mn SOD defend against oxidative damage by scavenging oxyradicals. Levels of Mn SOD and especially Cu/Zn SOD were significantly higher in corals heat stressed under PAR than in the dark, further evidence that elevated water temperatures caused oxidative stress (Downs *et al.* 2000, Woodley *et al.* 2001, Downs, Fauth & coauthors 2002). Independent researchers also concluded that heat stress causes oxidative stress, and leads to coral bleaching (Jones *et al.* 1998).

In 1999, coral colonies (*Montastraea annularis* species complex) sampled off Key Largo, FL, were exposed to elevated ocean temperatures for several months (Figure 1). There were strong positive correlations between oxidative damage and subsequent bleaching during the March-October sampling period (Woodley *et al.* 2001, Downs, Fauth & coauthors 2002). Corals with high levels of chlpHsp and SODs – typically colonies in shallower water – had less oxidative damage and did not bleach.

In contrast, corals with low chlpHsp and SODs – mostly those colonies in deeper water – experienced oxidative stress, had higher chaperonin levels (Hsp 60 and Hsp 70) and protein turnover activity, and bleached in late summer. Thus, bleaching was tightly coupled to the anti-oxidant and cellular stress capacity of coral colonies, suggesting bleaching was a final defense against oxidative stress (Downs *et al.* 2000, Woodley *et al.* 2001, Downs, Fauth & coauthors 2002).

This forecast exploits the time lag between temperature-induced cellular responses of corals (including upregulation of chlpHsp and antioxidant enzymes) and accumulation of damage products and subsequent

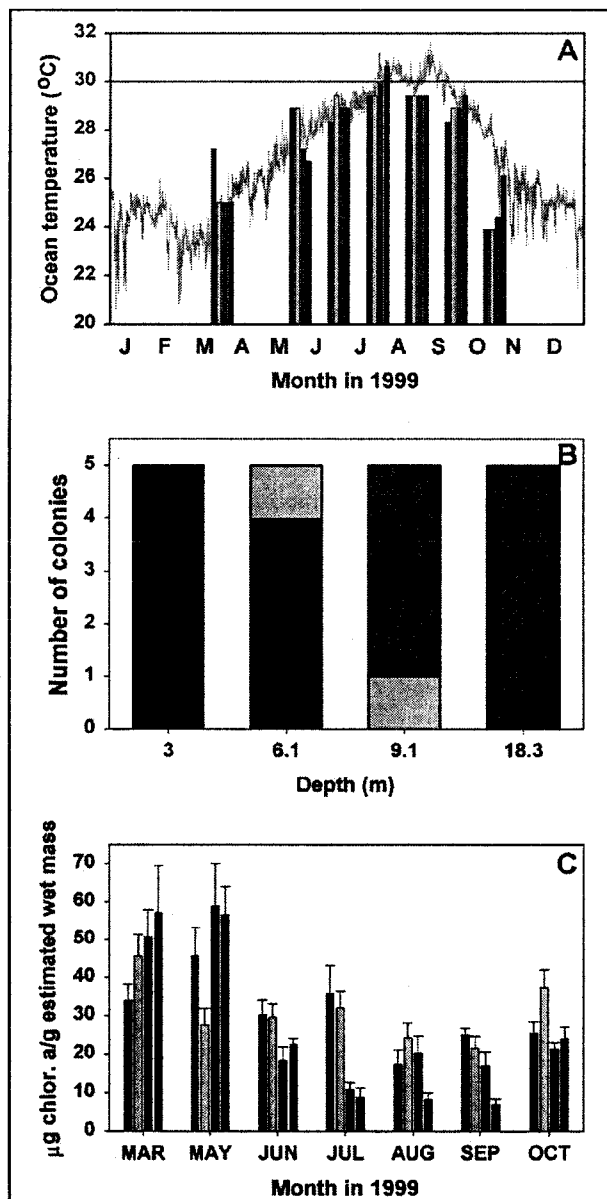


Figure 1. (A) Seasonal variation in ocean temperatures recorded beside coral colonies (*Montastraea annularis* species complex) sampled near Molasses Reef, off Key Largo, FL, USA. Bars show depth of colonies: black = 3.0 m, grey = 6.1 m, red = 9.1 m, blue = 18.3 m. Temperature beside the corals in summer differed significantly from all other months except May. Yellow line is mean sea surface temperature data recorded by a C-Man Buoy at Molasses Reef, 1-3 miles from our sites. Green line indicates the 30°C temperature threshold at which bleaching often occurs. (B) Colony condition in September, 1999 varied significantly among depths. Healthy colonies, black bars; paling colonies, gray bars; bleached colonies, red bars. (C) Chlorophyll a levels reflect photosynthetic activity and density of symbiotic zooxanthellae inhabiting coral tissue, providing a quantitative measure of bleaching. Bars show mean (± 1 SE) concentration of chlorophyll a in five coral colonies repeatedly sampled at each depth. Differences among months and the month $\times$ depth interaction were statistically significant (Woodley *et al.* 2001).

bleaching. Using logistic regression, the relationship between MDS parameters in spring and early summer and coral condition in September was examined. One biomarker sampled in March (chlpsHsp) explained significant variation in bleaching six months later, when bleaching became visible to SCUBA divers (Figure 2). The variation in bleaching explained by chlpsHsp levels (35 percent) was greater than that explained in an identical analysis using March ocean temperature (27 percent) as the independent variable. In both analyses, the proportion of variation explained by logistic regression was determined by the coefficient of determination (R^2).

Ocean temperatures continued rising through late spring. Corals in deeper water did not produce enough chlpsHsp to prevent oxyradicals from damaging cell components. Therefore, colonies in deeper water accumulated significantly more lipid peroxide (LPO) than colonies in shallower water (Woodley *et al.* 2001, Downs, Fauth & coauthors 2002).

May LPO levels also were significant predictors of bleaching (Logistic regression: $X^2 = 28.9$, $P < 0.0001$, 2 df, $R^2 = 0.76$). Colonies with <30 micromoles LPO/mg total protein in May were almost certain to be healthy in September, while those with greater damage were likely to pale or bleach.

As water temperatures continued increasing, the superoxide dismutases assayed – those produced only by the coral and not by their algal symbionts (Downs *et al.* 2000, Woodley *et al.* 2001, Downs, Fauth & coauthors 2002) – also could be used to forecast coral condition. June levels of Cu/Zn SOD, which localizes to the cytosol, explained variation in coral condition more than Mn SOD, which was restricted to the mitochondria (34 percent versus 19 percent, respectively). Colonies with high levels of these antioxidant enzymes were significantly less likely to pale or bleach than those with low antioxidant levels (Logistic regressions: both $X^2 = 7.33$, $P < 0.03$, 2 df). Thus, three separate biomarkers could be used to forecast coral bleaching, anywhere from three to six months in advance of sea surface temperature anomalies.

This forecasting method is in the early stages of development and testing. The next phase of the plan is to make the first specific, quantitative forecast of coral bleaching in 2002. Eventually, forecast could be used like hurricane forecasts – to alert resource managers

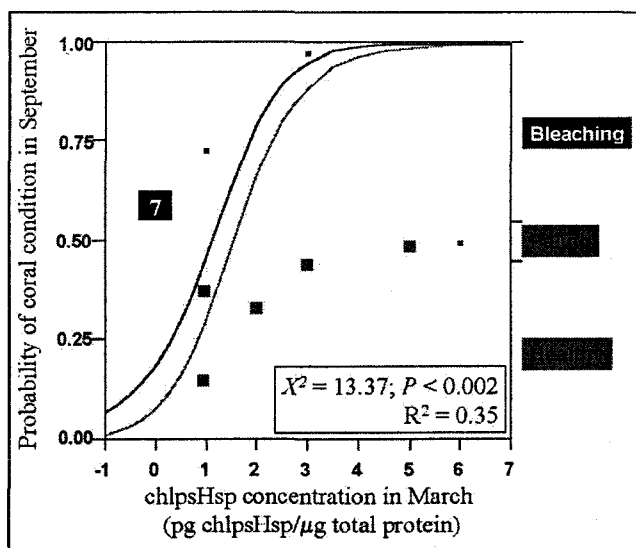


Figure 2. Cumulative logistic plot of the probability a coral colony was healthy (green), paling (red), or bleaching (black) in September, as a function of chloroplast small heat shock protein levels in March. Symbol size is proportional to the number of colonies with the same values. Smooth blue curves partition the probability axis among the three conditions, with healthy at lower right, bleaching at upper left, and paling in between. The probability of a colony having a specified condition when sea surface temperatures exceed 30° C for a prolonged period is given by the vertical distance between its probability curves. For example, the probability of a coral with 1 pg chlpsHsp in March being healthy six months later is 30 percent; of paling, 15 percent, and of bleaching, 55 percent.

and reef-based industries to the likelihood of a mass bleaching event in the upcoming year. Because the MDS allows identification of potential stressors, it also can be used to differentiate between global/regional and local forcing functions (i.e., climate warming and El Niño events versus point source pollution). Once sufficient data is obtained, it should be possible to forecast the effectiveness of different management options on coral bleaching.

Lessons Learned and Next Steps

The National Action Plan to Conserve Coral Reefs calls for a science-based ecosystem approach to coral reef conservation, and adaptive management to track and respond to environmental change and other anthropogenic threats (US Coral Reef Task Force 2000). This project includes close cooperation between a US NOAA/NOS biotechnology laboratory, US NOAA National Marine Sanctuaries managers, a private biotechnology company, and faculty from several colleges and universities.

The project has developed from an initial test of a promising new technology to a broader, more manage-

ment-based initiative. Recently a wider geographic region was sampled, including two more sites off Key Largo and another within Biscayne National Park. The expanded project includes additional sentinel species in the coral reef community: an alga (*Halimeda opuntia*), the corallivorous snail *Coralliophila abbreviata*, bicolor damselfish (*Pomacentrus partitus*), and white grunts (*Haemulon plumieri*).

Infrastructure is in place to transfer forecasts of coral bleaching from the research and development stage to the operational stage. EnVirtue Biotechnologies, Inc. created monospecific polyclonal antibodies against the coral cellular parameters discussed above, which substantially enhanced MDS resolution and precision. The new antibodies also require less coral tissue, minimizing damage to stressed colonies. EnVirtue Biotechnologies, Inc., also developed a robotic, high-throughput system which greatly reduces the time required for multiple assays on a large number of samples. Currently, acquiring samples using SCUBA divers and grinding the samples under liquid nitrogen in preparation for MDS assays are the most time-consuming steps.

While the forecasting model used here was simplistic, it improves on efforts based solely on sea surface temperature, which cannot predict small-scale or bathymetric variation in bleaching. Verifying the accuracy of MDS-based forecast requires obtaining more samples from diverse areas, and from additional species of corals, an effort already underway in the Florida Keys. The forecast also can be improved using multivariate statistical techniques, such as discriminant function and neural network analyses.

The most difficult steps remaining are 1) linking MDS parameters to other important life history traits of coral, such as growth, reproduction, survivorship, and susceptibility to disease, and 2) identifying the stressors impacting corals at different sites. Overcoming these hurdles will require vertically integrated sampling and experimentation, similar to what was successfully used to investigate declining amphibian populations (Collins *et al.* 2001).

Conclusions

There is an inverse relationship between expression of protective antioxidant/stress proteins and the incidence of oxidative damage and bleaching in boulder corals sampled within the Florida Keys National Marine Sanctuary, USA. Corals with high levels of antioxidants

and heat-shock proteins, which protect against the damaging effects of unusually high ocean temperatures, did not bleach. Those with low levels of protectant molecules suffered more oxidative damage, and were more likely to bleach (lose/expel their symbiotic algae). This correlation is similar to that observed in several human diseases (i.e., Alzheimer's disease, Parkinson's disease), where elevated levels of antioxidant and stress proteins protect cells from the degenerative influences of oxidative stress.

A simple regression procedure shows bleaching of individual coral colonies could be forecast months in advance from knowledge of specific biomarker levels. More sophisticated forecasts can be made using multiple markers and multivariate statistical techniques, but these will require larger data sets to test the accuracy of predictions than our logistic regression method.

Acknowledgements

Coral samples were collected under Florida Keys National Marine Sanctuaries and National Marine Fisheries Service permits. Statistical analyses were supported by NSF Grant DEB9727039 and EPA Grant R825795 to J. E. Fauth.

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Forecasting heat-induced coral bleaching: a molecular biomarker approach

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Running title: Forecasting coral bleaching

Keywords

bleaching, corals, ecological forecasting, El Niño, global warming, heat shock proteins, molecular biomarkers, reactive oxygen species, superoxide dismutases.

Type of article: Letter

146 words in abstract, 1461 words in full text; 1 table, 2 figures, 26 references

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BIOSKETCH

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Abstract

Mass coral bleaching is correlated with unusually high sea surface temperatures, but bathymetric patterns of bleaching often counter expected temperature and light gradients. We found expression of protective antioxidant/stress proteins was inversely related to both oxidative damage and the probability of bleaching in star boulder corals (*Montastraea annularis* species complex) during the 1999 El Niño event. Here we show that stress protein accumulations explain bathymetric patterns of bleaching, and can be used to forecast bleaching of individual coral colonies months before symptoms become visible. Colonies in shallow water accumulated heat shock proteins and superoxide dismutases that reduced oxidative damage during heat stress, while colonies at deeper sites did not, and were more likely to accumulate cellular damage products and to bleach. The results suggest molecular diagnostics can be used to identify bleaching-resistant coral colonies, which could be used to rebuild reefs damaged by rising ocean temperatures.

INTRODUCTION

Heat stress during El Niño events triggered mass coral bleaching, which devastated reefs worldwide during the past twenty years (Williams & Bunkley-Williams 1990; Goreau & Hayes 1994; Brown 1997; Stone *et al.* 1999). Currently, the U.S. National Oceanic and Atmospheric Administration (NOAA) uses unusually warm surface waters to identify regions where bleaching is likely (Hendee *et al.* 2001; NOAA National Environmental Satellite, Data, and Information Service 2002). However, during extreme sea-surface temperature anomalies, coral bleaching and mortality varies along spatial, temporal, bathymetric, size, and phylogenetic gradients (Williams & Bunkley-Williams 1990; Hughes & Tanner 2000; Hueerkamp *et al.* 2001; Loya *et al.* 2001; Obura 2001). Several authors comment on perplexing bleaching patterns: “it is a curious phenomenon that many intertidal corals . . . do not bleach, or bleach only partially and recover after a few months” (Stone *et al.* 1999); “paradoxically, juvenile *Acropora* colonies survived in the intertidal zone despite being exposed to high irradiance and desiccation during summer midday low tides” (Loya *et al.* 2001). While bleaching is likely in regions that experience high sea-surface temperatures in late summer, predicting which individual colonies bleach remains a challenge.

Here, we demonstrate cellular-physiological parameters can forecast temperature-induced bleaching of individual corals 2-6 months before distress becomes visible. Our method exploits time lags between rising ocean temperatures, increased production of heat shock proteins and antioxidant enzymes, and accumulation of cellular damage products and subsequent bleaching. Coral colonies exposed to elevated temperatures display several cellular defenses. First, their symbiotic zooxanthellae produce chloroplast small heat shock protein (chlpsHsp), which protects Photosystem II activity (Downs *et al.* 2000, 2002). When this defense is overwhelmed, oxyradicals are generated, which are scavenged by superoxide dismutases produced by both zooxanthellae and their cnidarian hosts. However, when these enzyme systems are overwhelmed, reactive oxygen species cause lipid peroxidation, protein degradation, DNA damage, bleaching, and ultimately tissue and cell death (Hawkrigde *et al.* 2000).

Because cellular defenses vary among colonies (Downs *et al.* 2002), it is possible to forecast which corals within ocean “hot spots” are likely to bleach.

METHODS

Coral sampling

We sampled boulder star corals [*Montastraea annularis* species complex (Ellis and Solander)] from four sites (3, 6, 9, and 18 m depths) near Molasses Reef, Florida Keys National Marine Sanctuary, USA. Boulder star corals are dominant reef-building species, and exhibited a bleaching gradient with depth in 1997-1998; colonies in shallow water generally did not bleach, while those in deeper water bleached and sometimes died. We collected samples in March, and monthly May - October, 1999, from five colonies at each site. Divers wrapped samples in aluminum foil and placed them in bags underwater. They also recorded ocean temperature, and scored colony condition as healthy (normally pigmented), paling (diminished pigmentation), or bleaching (zooxanthellae densities greatly reduced, revealing the white skeleton beneath). On deck, water was removed and samples frozen using liquid N₂. Samples were stored at -80° C until analysis by EnVirtue Biotechnologies, Inc.

Immunoassays

We assayed eleven cellular and molecular parameters, as described in Downs *et al.* (2002). Briefly, we used spectrophotometric methods (Heckathorn *et al.* 1998; Downs *et al.* 2000) to assay lipid peroxidation (LPO) and total glutathione (GSH), and enzyme-linked immunosorbent assays (Robinson *et al.* 1999, Downs *et al.* 2002) for total protein carbonyl, total ubiquitin, cnidarian Cu/Zn and Mn superoxide dismutases (SODs), cnidarian heat shock proteins (Hsp) 60 and 70, cnidarian ubiquitin, and dinoflagellate chlpsHsp and mitochondrial small heat shock protein (sHsp). Using an integrated suite of molecular biomarkers allowed us to separate cnidarian and zooxanthellae stress responses (Downs *et al.* 2000, 2002; Woodley *et al.* 2001).

Statistical analyses

We used logistic regression (Sokal & Rohlf 1995) to examine the relationship between cellular parameters recorded each month, and coral condition in September. Regressions were considered statistically significant if $P < 0.05$, and biologically relevant if coefficients of determination (R^2) exceeded that of regressions using ocean temperature as the independent variable. We used canonical discriminant analysis (SAS Institute, Inc. 1996) to delineate boundaries among coral condition based on mean parameter values averaged from March - August ($n = 20$). All statistical analyses were performed using JMP V. 4.0 (SAS Institute, Inc., Cary, NC).

RESULTS

Bathymetric pattern of bleaching

Sea-surface temperature exceeded 30° C for prolonged periods in August and September (Woodley *et al.* 2001), and bleaching increased in frequency with increasing depth (Fig. 1). No colonies bleached at the 3 m site, but one paled at 6 m. In contrast, four colonies bleached and one paled at the 9 m site, and all colonies at 18 m bleached - including one that later died. All nineteen other colonies recovered by March 2000 (Downs *et al.* 2002; Fauth *et al.* in press).

Forecasting coral bleaching

Eight cellular parameters explained more variation in subsequent colony condition than did ocean temperature (Table 1). March accumulations of chlpsHsp and GSH were significant predictors of coral condition six months later. Colonies with modest amounts of protective chlpsHsp and low GSH levels were least likely to bleach (Fig. 1A, B). May LPO accumulations explained >75% of the variation in coral condition four months later. Corals with little LPO, which assays oxidative damage to lipid membranes, were least likely to bleach (Fig. 1C). The only parameter that explained more variation was July sHsp (Table 1). Corals with substantial accumulations of sHsp, which in higher plants protects the NADH:ubiquinone oxidoreductase complex of oxidative phosphorylation

(Downs & Heckathorn 1998; Hamilton & Heckathorn 2001), were least likely to bleach two months later (Fig. 1D). Other predictors of coral bleaching included four cnidarian parameters: both SODs, Hsp 60, and α B-crystallin (Table 1).

Canonical discriminant analyses showed chlpsHsp, sHsp, and Cu/Zn SOD distinguished between bleached and unbleached corals, while paling colonies were distinguished by LPO (Fig 3). Good separation among groups verified that molecular parameters could diagnose coral condition.

DISCUSSION

The National Action Plan to Conserve Coral Reefs (U.S. Coral Reef Task Force 2000) called for adoption of a science-based, ecosystem approach to coral reef conservation, and adaptive management to track and respond to environmental change and other anthropogenic threats. Development of expert systems and remote-sensing methods to identify ocean “hot spots” was an important step in identifying regions with environmental conditions conducive to bleaching (Hendee *et al.* 2001; Carriquiry *et al.* 2001). However, even within “hot spots” bleaching frequency varies with coral species, depth, size, and microenvironmental factors (Marshall & Baird 2000; Loya *et al.* 2001, Brown *et al.* 2002). Our goal was to develop a molecular-diagnostic method to forecast bleaching of individual corals under natural conditions.

Previously, we reported strong correlations between accumulation of protectant molecules and oxidative damage products, and subsequent bleaching of boulder star corals throughout the 1999 El Niño event (Woodley *et al.* 2001; Downs *et al.* 2002). Bleaching was intimately coupled to the anti-oxidant and cellular stress capacity of coral colonies. These strong correlations suggested molecular diagnostics could be used to forecast coral bleaching. Retrospective analyses showed two parameters we measured (chlpsHsp and GSH) could predict coral condition six months in advance. Two other parameters, May LPO and July sHsp, explained >75% of the variation in September coral condition. Coefficients of determination (R^2) for logistic regressions with molecular parameters were 7-30% larger than for models with ocean temperature, illustrating the

utility of this mechanistic approach.

Although these results apply only to heat-induced bleaching in boulder star corals, preliminary analyses suggest molecular diagnostics can distinguish between environmental and pathogen-induced bleaching (Woodley *et al.* 2001). Similar assays conducted on other coral genera, including deep-water *Oculina varicosa* (Woodley *et al.* 2001), and Indo-Pacific *Goniastrea aspera* (Brown *et al.* 2002), demonstrate molecular diagnostics can be applied to corals worldwide. Doing so requires quantifying nominal parameter levels for unstressed corals, and determining the relationship between stress levels and life-history responses.

Our results show molecular-diagnostic methods can be used to forecast heat-induced coral bleaching. Combined with data on sea-surface temperature (Hendee *et al.* 2001), quantitative forecasts can be used in much the same manner as hurricane forecasts – to alert resource managers and reef-based industries to probable mass bleaching events. Sampling multiple colonies within a site permits estimation of both mean and uncertainty of predicted outcomes, two key components of ecological forecasts (Clark *et al.* 2001). Because our molecular diagnostic method permits identification of potential stress agents (Woodley *et al.* 2001), it also can be used to differentiate between global/regional and local forcing functions (i.e., climate warming and El Niño events versus point source pollution). Given sufficient data, it should be possible to forecast the effectiveness of different management options on coral bleaching. Perhaps most importantly, molecular-diagnostics could be used to identify coral colonies resistant to bleaching (see Dustan 1982; Brown *et al.* 2002), which therefore would be desirable candidates for reef restoration.

ACKNOWLEDGEMENTS

Coral samples were collected under Florida Keys National Marine Sanctuaries and National Marine Fisheries Service permits. Statistical analyses were supported by NSF Grant DEB9727039 and EPA Grant R825795 to J. E. Fauth.

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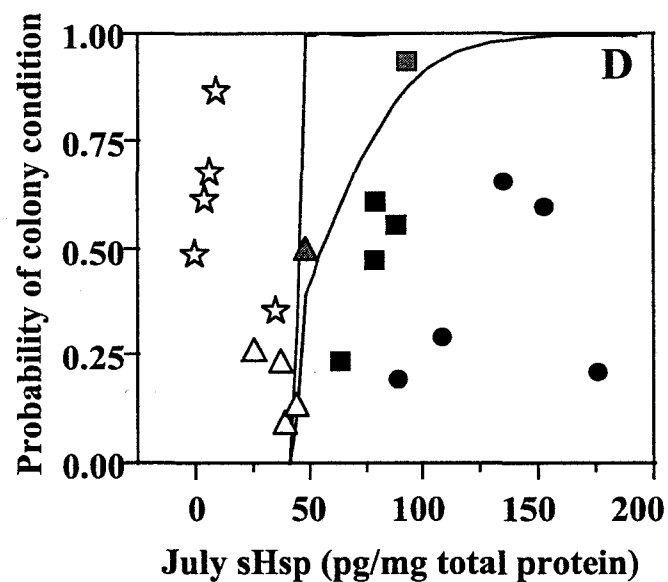
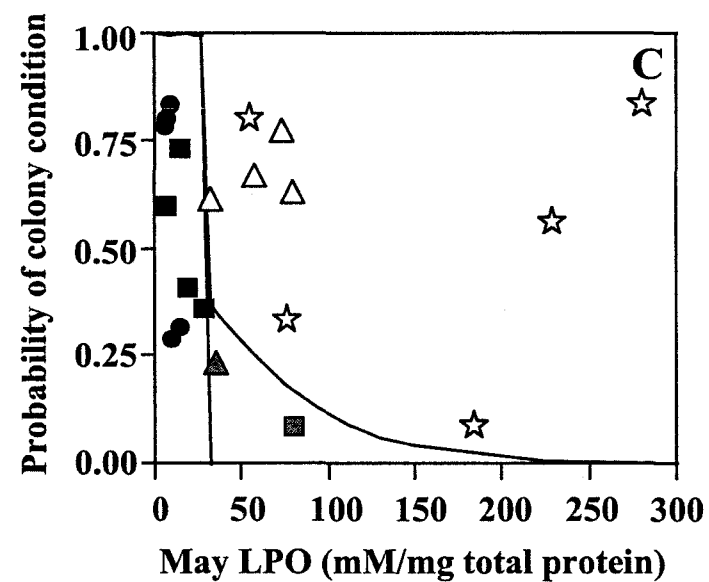
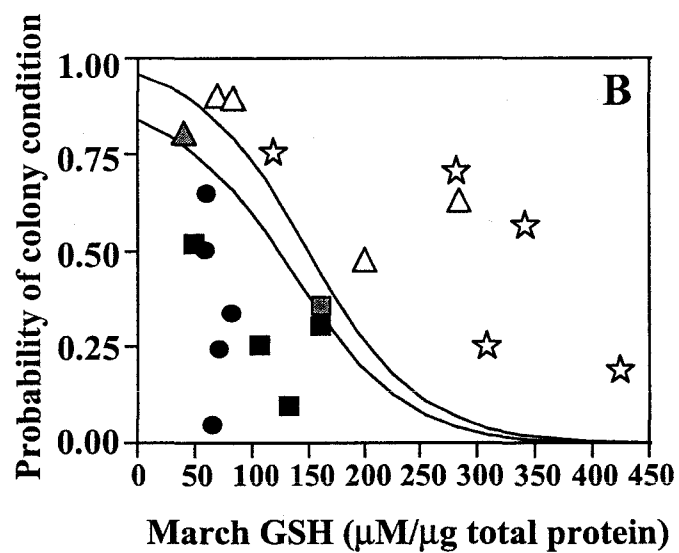
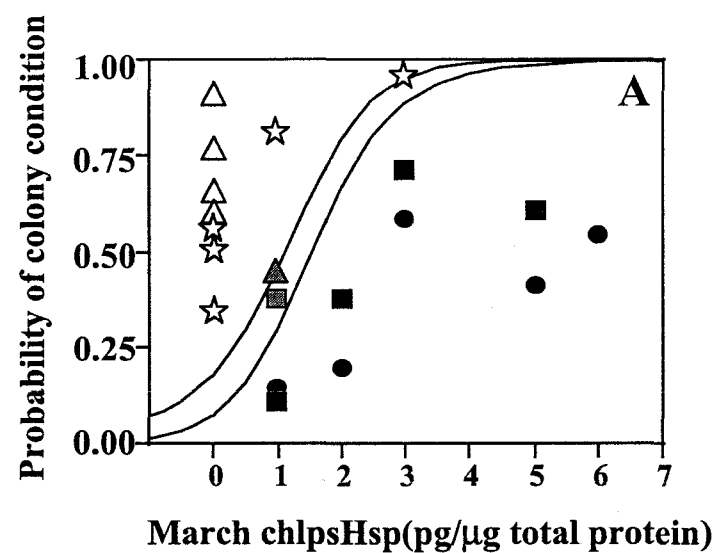
Table 1 Cellular parameters associated with bleaching of star boulder corals (*Montastraea annularis* sp. complex) sampled near Molasses Reef, FL, U.S.A. Logistic regressions tested whether cellular parameters assayed in spring and early summer explained more variation in coral condition (healthy, paling, or bleaching) in September than did ocean temperature, which is currently used to forecast coral bleaching. Parameters: chloroplast small heat shock protein, chlpsHsp; total glutathione, GSH; lipid peroxide, LPO; superoxide dismutase, SOD; small heat shock protein, sHsp; heat shock protein, Hsp).

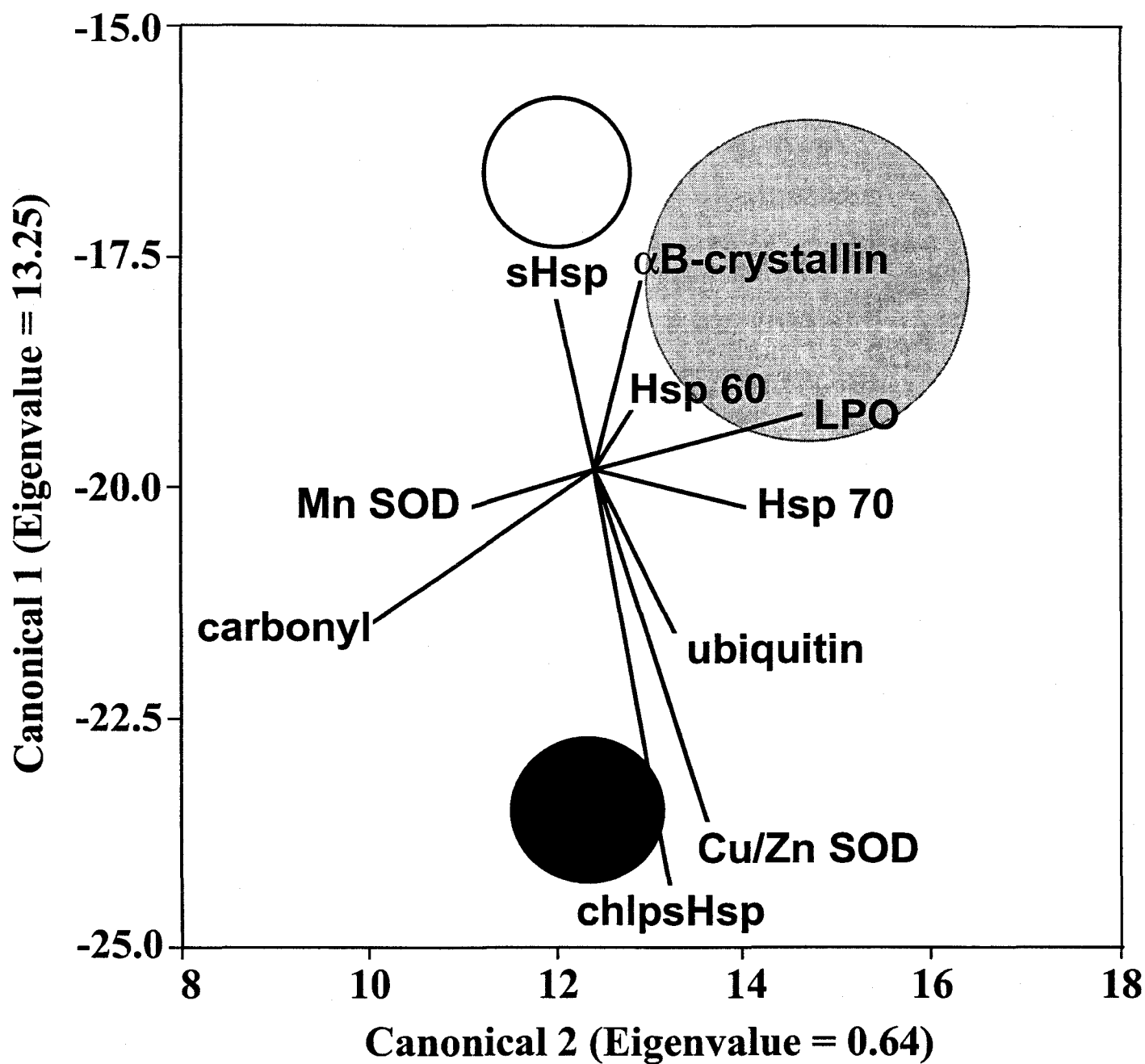
Month	Parameter	χ^2 value	Significance (p)	R^2	Ocean temp. R^2
March	chlpsHsp	13.4	0.0012	0.35	0.27
	GSH	10.8	0.0044	0.29	
May	LPO	28.9	0.0001	0.76	0.70
June	Cu/Zn SOD	12.8	0.0017	0.34	0.04
	sHsp	9.6	0.0082	0.25	
	Mn SOD	7.3	0.0257	0.19	
July	sHsp	29.9	0.0001	0.79	0.70
August	sHsp	23.3	0.0001	0.61	0.00
	Cu/Zn SOD	20.7	0.0001	0.55	
	chlpsHsp	18.9	0.0001	0.50	
	α B-crystallin	13.2	0.0014	0.35	
	Hsp 60	8.6	0.0137	0.23	
	GSH	6.8	0.0341	0.18	

Figure legends

Figure 1 Cumulative logistic plots of the probability a coral colony was healthy (solid symbols), paling (shaded symbols), or bleaching (open symbols) in September, as a function of (A) chloroplast small heat shock protein (chlpsHsp) and (B) total glutathione (GSH) accumulations in March, (C) lipid peroxide (LPO) in May, and (D) total small heat shock protein (sHsp) in July. Curves partition the probability axis among the three conditions; the probability of a colony having a specified condition when sea-surface temperatures exceed 30° C for a prolonged period is given by the vertical distance between its probability curves. For example, the probability of a coral with 1 pg chlpsHsp in March being healthy six months later is 30%; of paling, 15%; and of bleaching, 55%. Symbols show colony depth: circles, 3 m; squares, 6 m; triangles, 9 m; stars, 18 m.

Figure 2 Canonical centroid plot of mean parameter values prior to coral bleaching. Circles are 95% confidence regions: healthy colonies, solid; paling colonies, shaded; bleached colonies, open circles. Differences among coral conditions were statistically significant (Wilks' $\lambda = 0.0415$, $F_{14,22} = 2.49$, $p < 0.041$). Biplot rays show directions of original parameters within canonical space. Canonical correlations adjusted for differences among group means (partial correlations, *pcorr*) showed chlpsHsp, sHsp and Cu/Zn SOD distinguished unbleached colonies from those that bleached or paled (*pcorr* = -0.41, -0.33, and -0.31, respectively), while LPO distinguished paling colonies (*pcorr* = +0.43).





Coastal Monitoring Through Partnerships

Proceedings of the Fifth Symposium on the
Environmental Monitoring
and Assessment Program (EMAP)

Pensacola Beach, Florida, U.S.A. April 24-27, 2001



Scientific Editors
Brian D. Melzian, Virginia Engle,
Malissa McAlister and Shabeg Sandhu

Kluwer Academic Publishers

FORAMINIFERA AS BIOINDICATORS IN CORAL REEF ASSESSMENT AND MONITORING: THE FORAM INDEX

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Abstract. Coral reef communities are threatened worldwide. Resource managers urgently need indicators of the biological condition of reef environments that can relate data acquired through remote-sensing, water-quality and benthic-community monitoring to stress responses in reef organisms. The "FORAM" (Foraminifera in Reef Assessment and Monitoring) Index (FI) is based on 30 years of research on reef sediments and reef-dwelling larger foraminifera. These shelled protists are ideal indicator organisms because:

- Foraminifera are widely used as environmental and paleoenvironmental indicators in many contexts;
- Reef-building, zooxanthellate corals and foraminifera with algal symbionts have similar water-quality requirements;
- The relatively short life spans of foraminifera as compared with long-lived colonial corals facilitate differentiation between long-term water-quality decline and episodic stress events;
- Foraminifera are relatively small and abundant, permitting statistically significant sample sizes to be collected quickly and relatively inexpensively, ideally as a component of comprehensive monitoring programs; and
- Collection of foraminifera has minimal impact on reef resources.

USEPA guidelines for ecological indicators are used to evaluate the FI. Data required are foraminiferal assemblages from surface sediments of reef-associated environments. The FI provides resource managers with a simple procedure for determining the suitability of benthic environments for communities dominated by algal symbiotic organisms. The FI can be applied independently, or incorporated into existing or planned monitoring efforts. The simple calculations require limited computer capabilities and therefore can be applied readily to reef-associated environments worldwide. In addition, the foraminiferal shells collected can be subjected to morphometric and geochemical analyses in areas of suspected heavy-metal pollution, and the data sets for the index can be used with other monitoring data in detailed multidimensional assessments.

Keywords: bioindicators, coral reefs, Foraminifera, zooxanthellae, algal symbiosis

1. Introduction

Human activities are changing environmental conditions on a global scale. Roughly half the Earth's land area has been transformed or degraded (Vitousek *et al.*, 1997). Human activities have effectively doubled the annual transfer of nitrogen from the atmospheric pool of N₂ to biologically available fixed nitrogen (Schnoor *et al.*, 1995). Much of this fixed nitrogen, along with nitrous oxide gases from burning of fossil fuels (Prinn *et al.*, 1990), is washed into aquatic systems by rain. As a result of strato-



spheric ozone depletion, the intensity of biologically damaging ultraviolet radiation (UV_B) at 20° N latitude between April and August now exceeds the June 1969 (summer solstice) maximum (Shick *et al.*, 1996). Carbon dioxide concentration in the atmosphere has increased by nearly 30% since the beginning of the Industrial Revolution (Shimel *et al.*, 1995), with impacts ranging from global climate change to changes in ocean chemistry that inhibit calcification.

The U.S. Environmental Protection Agency's (EPA) Environmental Monitoring and Assessment Program (EMAP) was created to develop and monitor indicators of pollution exposure and habitat condition. Objectives are to determine the magnitude and geographic distribution of resources that are adversely impacted by pollution and other environmental stresses (Messer *et al.*, 1991). Coral reefs are among the ecosystems most threatened by human activities. Bryant *et al.* (1998) estimated that nearly 60% of the Earth's coral reefs are threatened by relatively local impacts including nutrients and other chemical pollutants, sedimentation, destructive fishing practices, and shipping. An assessment of the status of the world's coral reefs in 2000 concluded that more than one quarter have been lost (Wilkinson, 2000), about 15% to mortality following 1997–98 mass bleaching events. Risk (1999) noted that resource managers have monitored water quality and reef conditions for decades but they lack bioindicators that can link those measures to meaningful efforts to preserve remaining reef resources. Attempts to interpret water-quality data continue to be confounded by the reef community's ability to sequester nutrients, making them unavailable to monitoring yet readily effective in inducing community change, an enigma recognized by Laws and Redalje (1979) but widely misunderstood (Risk, 1999).

This paper utilizes EPA "Evaluation Guidelines for Ecological Indicators" (Jackson *et al.*, 2000) to present a simple index, the FORAM (Foraminifera in Reef Assessment and Monitoring) Index (FI), based on foraminiferal assemblages from surface sediments. The FI is intended to provide resource managers with a measure, which is independent of coral populations, to determine whether water quality in the environment is sufficient to support reef growth or recovery. A major advantage of foraminifera in reef assessment is their short life span, as compared with long-lived reef-building corals. Foraminiferal assemblages can potentially facilitate differentiation between long-term reef decline associated with declining water quality and temporary reef decline associated with episodic mortality events (Cockey *et al.*, 1996).

2. Conceptual Relevance

2.1 RELEVANCE TO ASSESSMENT

The FI applies historic observations that healthy coral reefs had abundant mixotrophic larger foraminifera (e.g., McKee *et al.*, 1956; Hallock 1981a, 1988), which were important sediment constituents. On coral reefs that are subject to

significant nutrification (i.e., increase in nutrient flux that results in change in biological community structure), with or without increased terrigenous sedimentation, populations of smaller heterotrophic foraminifers proliferate and their shell numbers overwhelm those of declining larger foraminifers (Hirshfield *et al.*, 1968; Cockey *et al.*, 1996).

Unfortunately, over the past 25 years, reef-building corals have declined nearly worldwide in response to a variety of factors unrelated to nutrification, including new diseases, bleaching in response to temperature stress, and physical impacts such as hurricanes and ship groundings. Coral reef communities are subject to a myriad of stresses and are declining from most of them. Thus, it is critical to have an indicator of water-quality conditions that will support reef development, even in the absence of healthy coral populations following mass mortality events. Cockey *et al.* (1996) argued that larger foraminiferal populations, which are immune to coral-specific diseases and recover much more quickly from physical impacts than long-lived coral populations, are sensitive indicators of water-quality conditions that support reef development.

According to Engle (2000, p. 3-1), "An ideal indicator of the response of benthic organisms to perturbations in the environment would not only quantify their present condition in ecosystems but would also integrate the effects of anthropogenic and natural stressors on the organisms over time (Boesch and Rosenberg 1981; Messer *et al.* 1991)." This information is precisely what foraminiferal tests in the sediments provide.

2.2 RELEVANCE TO ECOLOGICAL FUNCTION

Environmental perturbations of critical concern to coral reefs fall into three major categories (e.g., Hallock 2000; 2001): local impacts, new diseases of regional extent, and global change. The FORAM Index can be used to address local impacts and to assist in differentiating between local impacts that affect water quality and impacts that result from regional- to global-change issues.

The principal physiological analogy between reef-building corals and larger foraminifers is the dependence of both groups on algal symbionts to enhance growth and calcification (e.g., Lee and Anderson, 1991). This analogy and examples from both groups were used to develop a model to predict the energetic benefits of algal symbiosis (Hallock, 1981b), with the conclusion that this mode of life was energetically most advantageous when dissolved nutrients (i.e., NH_4^+ , NO_2^- , NO_3^- , PO_4^{3-}) and particulate food resources were scarce. Physiological studies of corals (e.g., Falkowski *et al.*, 1993; Steven and Broadbent, 1997) and of larger foraminifers (Lee, 1998) have since demonstrated that fixed nitrogen limitation is crucial to maintenance of the host-symbiont relation.

Birkeland (1977; 1988) recognized that nutrient availability on the local or regional scale is a major control on benthic-community structure, especially in sub-

tropical and tropical seas (see also Hallock and Schlager, 1986; Hallock *et al.*, 1993). Coral reefs thrive in the most nutrient-depleted oceanic waters where mixotrophic nutrition, i.e., the recycling of nutrients between host and algal symbionts, is most advantageous. As the nutrient supply increases, reef-building coral domination of the benthos gradually gives way to macroalgal domination as algal symbiosis becomes less advantageous. Slightly higher nutrient flux promotes phytoplankton blooms in the water column, limiting light penetration to the benthos. Light attenuation promotes the dominance of the benthos by non-symbiotic filter-feeding animals such as sponges and ascidians and of detritus-feeding echinoderms and crustaceans that do not directly require sunlight for survival (as observed, e.g., in Kaneohe Bay, Hawaii by Smith *et al.*, 1981).

Benthic foraminiferal assemblages respond similarly to nutrient flux (Hallock, 1987, 1988). In very low-nutrient marine environments, such as those found around most Pacific atolls, larger foraminifers that host algal endosymbionts (Table 1) completely dominate sand-size sediments in reef systems (e.g., McKee *et al.*, 1956; Hallock, 1981a). As nutrient supplies increase, bioeroded coral fragments, calcareous algae, molluscan debris, and smaller herbivorous and detritivorous foraminifers (Table 1) become more common as sediment constituents (Hirschfield *et al.*, 1968; Hallock, 1988; Cockey *et al.*, 1996).

As the environment becomes unsuitable for the survival of foraminifers with algal symbionts, their dead tests become rare in the sediments, and remnants become increasingly corroded (e.g., Cottey and Hallock, 1988). These changes occur with nutrification, which does not result in a measurable increase in dissolved nutrients in the water column because the planktic and benthic communities are able to incorporate and utilize all available nutrients (e.g., Laws and Redalje, 1979). True "eutrophication," which is nutrification to the degree that organic carbon buildup occurs in bottom waters and sediments (e.g., Cockey *et al.*, 1996), results in further change in benthic-community structure including domination by opportunistic taxa (Table 1) that can tolerate episodic anoxia (e.g., Alve, 1995).

A critical application of the FI is differentiation between nutrification-induced decline in coral dominance in a reef environment and decline in response to episodic stress or mortality events (e.g., temperature extremes or hurricanes) that are independent of water quality. While the immediate cause of coral population decline is often a mortality event, if chronic nutrification has also occurred, coral populations continue to decline rather than recover from that event.

3. Methods

3.1 SAMPLE COLLECTION AND PROCESSING

Several sample collection procedures have been successfully used by numerous researchers who have assessed foraminiferal assemblages in surface sediments. For example, samples can be collected by SCUBA divers using a scoop and plas-

Table 1. Functional Groups of Foraminifers Used in Coral Reef Assessments

Functional Group	Order	Family	Genus	Distribution
Symbiont-Bearing	Rotaliida	Amphisteginidae	<i>Amphistegina</i>	Circumtropical
		Calcarinidae	5 genera	Indo-Pacific
		Nummulitidae	<i>Heterostegina</i>	Circumtropical
	Miliolida	Alveolinidae	3 other genera	Indo-Pacific
			<i>Alveolinella</i>	Indo-Pacific
		Peneroplidae	<i>Borelis</i>	Circumtropical
			Several genera	Circumtropical
			<i>Sorites</i>	Circumtropical
			<i>Amphisorus</i>	Circumtropical
			3 genera	Caribbean
			<i>Marginopora</i>	Indo-Pacific
Opportunistic*	Trochamminida	Trochamminidae	Several genera	Cosmopolitan
	Textulariida	Lituolidae	Several genera	Cosmopolitan
	Buliminida	Bolivinidae	Several genera	Cosmopolitan
		Buliminidae	Several genera	Cosmopolitan
	Rotaliida	Rotaliidae	<i>Ammonia</i>	Cosmopolitan
		Elphidiidae	<i>Elphidium</i>	Cosmopolitan
Other Small Taxa	Miliolida	Most except larger taxa noted above		Cosmopolitan
	Rotaliida	Most except those noted above		Cosmopolitan
	Textulariida	Most		Cosmopolitan
	Other	Most		Cosmopolitan

*Full range of opportunistic genera under local conditions is not well known.

tic bags (e.g., Donnelly, 1993) or minicore (e.g., Cockey *et al.*, 1996). When grab samples or box cores are routinely taken for invertebrates and/or sediment analyses in larger monitoring efforts (e.g., McRae *et al.*, 1998; Engle, 2000), a small surface subsample (upper 2 cm) for foraminiferal analysis can simply be processed separately.

The ideal sample size is 10 grams dry weight, which is a volume of approximately 10–20 cm³. A sample of this size can be split in half, with one half saved as backup or archived. The other half is divided into an approximately 1 gram portion and a 4 gram portion. The 4 gram portion is used for routine sediment grain-size analysis (Folk, 1974). If such analysis is being performed as part of the larger monitoring effort, then it need not be repeated on the foraminiferal sample.

The 1-gram portion of the sample is washed with fresh water over a 63-μm mesh sieve to remove mud-size sediments, then dried on filter paper at 40–50°C until the sample is thoroughly dry. The dried sample is gently disaggregated, thoroughly mixed, and poured into a mound on a clean, smooth surface. With a fine spatula, a small scoop of the sample (approximately 0.1 g) is removed from the center of the mound and weighed to the nearest milligram. The weighed subsample is sprinkled over a small,

gridded tray and examined using a conventional stereomicroscope or a video-imaging system. A very fine artist's brush (tip size 3/0 to 5/0), moistened with water, is used to remove foraminiferal specimens from the sediment (heavily worn and reworked specimens are excluded). Each specimen is placed onto a cardboard micropaleontological faunal slide, which is lightly coated with water-soluble glue. Then a preliminary count is made of each individual. If the number of foraminiferal specimens is approximately 150–200, the subsample is sufficient. If fewer specimens were present in the first portion, then a second portion is removed from the mound, weighed, and sorted. This procedure is repeated until 150–200 specimens are obtained or until the entire gram of sample is processed.

3.2 DATA ANALYSIS AND INFORMATION MANAGEMENT

Once an adequate subsample is obtained, the foraminifers are sorted by genus and counted. Generic-level identification is recommended because it is historically well established and an excellent basic reference is available (Loeblich and Tappan, 1987); species-level identifications tend to be inconsistent across investigators. If deformed specimens are observed, the proportions of deformed specimens of abundant taxa should also be noted. Deformed foraminifers are well-known indicators of heavy-metal pollution (Alve, 1995; Yanko *et al.*, 1998).

Raw counts are entered onto a spreadsheet, with appropriate sample location and identification codes, environmental data, and the weight of the sample picked. Basic data from the counts include relative abundance (proportions of the subsample) and absolute abundance (# specimens/gram of sediment) of each genus identified. Information management should be standardized to that of the larger monitoring program (e.g., McRae *et al.*, 1998; Engle, 2000). As resources permit, data analyses such as multivariate and multidimensional analyses can be performed consistent with those applied to other data sets from the monitoring program.

Procedures for calculating the FI are presented in Table 2. Foraminiferal relative-abundance data are summed into functional groups, which include taxa of larger foraminifers that host algal symbionts, pollution-tolerant opportunistic foraminifers that dominate high-stress environments, and small taxa that proliferate in response to nutrification. The basic premise upon which this index is based is that environments suitable for proliferation of symbiont-bearing organisms have sediments in which at least 25–30% of the foraminiferal tests were produced by taxa that hosted algal symbionts. A sample that contains 25% larger foraminiferal tests and 75% tests of other small taxa has a FI = 4. Environments with sediments devoid of larger foraminiferal tests by definition have a FI $\leq$ 2. FI values between 2 and 4 in sediments from areas with existing coral reefs indicate that conditions are marginal to unsuitable for recovery of coral communities after a mortality event. Several of the data sets upon which these interpretations are based are presented and discussed in Section 4.

Table 2. Calculating the FORAM Index (FI)

Step 1. From each subsample examined, sort all foraminiferal specimens by genus, count, and record in a spreadsheet, with genera arranged by functional group. (See Table 1.)

Step 2. Calculate the proportion (P) of specimens for each functional group by summing the specimens of each genus of that group (N) and dividing by the total number of specimens counted (T).

- a) $P_s = N_s/T$, where subscript "s" represents symbiont-bearing foraminifers
- b) $P_o = N_o/T$, where subscript "o" represents opportunistic foraminifers
- c) $P_h = N_h/T$, where subscript "h" represents other small, heterotrophic foraminifers

Step 3. Weight proportions to calculate the FORAM Index (FI):

$$FI = (10 \times P_s) + (P_o) + (2 \times P_h)$$

Step 4. Interpretation:

- FI > 4 indicates environment conducive to reef growth
 - FI varying between 3 and 5 indicates environmental change (Coefficient of Variation > 0.1)
 - $2 < FI < 4$ indicates environment marginal for reef growth and unsuitable for recovery
 - FI < 2 indicates stressed conditions unsuitable for reef growth
-

3.3 QUALITY ASSURANCE

Foraminifers can be used as benthic indicators in new or existing monitoring programs that include routine sampling for analysis of sediment parameters (e.g., texture, chemical pollutants, nutrients) and/or benthic invertebrates. Basic Quality Assurance and Quality Control (QA/QC) procedures developed for field sampling, laboratory processing and data analysis for benthic data under EMAP guidelines are ideal (e.g., Engle, 2000). Foraminiferal assemblage analysis is relatively insensitive to the method of sediment collection employed, although it is important to collect the sediment surface layer, and it is important that the surface layer be undisturbed until it is sampled. Whatever collection method is employed, the sampling team should be instructed in the technique and should use it consistently.

To ensure comparability of taxonomic identification, the scientist or technician performing the identifications should be trained and supervised by a specialist in benthic foraminiferal identification, and QA/QC procedures should be employed (e.g., Engle, 2000). For monitoring efforts in remote areas or developing countries where resources are severely limited, technicians can be trained and selected samples can be sent to a specialist at a local university or even in another country to check for consistency in identifications.

3.4 MONETARY COSTS

The monetary costs of collecting foraminifers in sediment samples depends upon the type of monitoring program into which this procedure is being incorporated.

If grab samples or box cores are already being collected for benthic invertebrates and sediment characterization, the per-sample cost to include foraminiferal samples is minimal. Supplies required are small plastic bags or vials and labels. A maximum of 5-10 minutes/sample is needed for label preparation, sample collection, sample sealing, and sample recording. The major cost of including foraminiferal assemblages in monitoring studies is the cost of a technician to analyze samples. The procedure described above provides not only data needed for the FI, but also assemblage data that can be incorporated in more extensive multidimensional analyses. Using this procedure, one dedicated technician can analyze up to three samples per day, depending upon other responsibilities. If screening of large numbers of samples is a priority for the project, an experienced technician can directly count the foraminifers into the three functional groups described above without separating specimens onto slides, roughly tripling the number of samples that can be processed per day. These samples can be economically stored for later detailed analyses as time and resources permit.

Preferred minimum qualifications for the foraminiferal analyst are a Bachelor's degree in a biological or geological science with some experience in taxonomic identification and statistical analysis; Master's-level education with specialized training in foraminiferal ecology is ideal. Basic laboratory needs include a stereomicroscope, a computer to routinely enter data, taxonomic resources (i.e., Loeblich and Tappan, 1987, and local references), and assorted supplies. Recurring costs include supplies and computer and software upgrades.

The FI can be economically adopted in developing countries. Slides and holders can be made at minimal cost from glue, paint, recycled cardboard and aluminum beverage cans (Bayu Ludvianto, Personal Communication). Samples can be collected from a canoe or raft, if necessary, using a small grab, which can be locally fabricated, or by a proficient snorkel or SCUBA diver. Because data required for the FI are simple counts, basic calculations and graphics can be done by hand or with an inexpensive hand calculator. A computer is required only to archive and analyze data further. A researcher at a field laboratory could easily perform sample collection, microscopic analysis, data recording and FI calculations. Data sheets can then be sent to an in-country research center or university or to a project coordinator in another country for more detailed analyses of data sets. Also, because unprocessed samples are relatively small and do not require refrigeration, they can be shipped relatively inexpensively from a field location to a laboratory for processing and analysis.

4. Response Variability

4.1 SAMPLE SIZE

Historically, most foraminiferal researchers have counted 300 specimens per sediment sample to obtain data for community analyses. To determine if smaller sample sizes provide consistent data, Dix (2001) used the basic procedure outlined previ-

ously to pick three subsamples of approximately 100 foraminifers per subsample from each of 12 samples. Each subsample was picked, the foraminifers identified to genus, and the relative abundances of each genus calculated for that subsample. The raw data from each set of three subsamples were pooled and used as the "expected" distribution for relative abundances. The Kolmogorov-Smirnov Goodness of Fit test was used to compare all pairs of subsamples, as well as all individual subsamples against their respective pooled "expected" distribution. Out of the 72 resultant comparisons, only one pair of subsamples was significantly different at the 0.05 probability level. Dix concluded that, for generic identifications, a sample size of 150-200 provided a useful compromise between the added precision of larger samples (i.e., 300) and added cost of processing (i.e., double the time of picking and identification).

4.2. SPATIAL AND TEMPORAL VARIABILITY

Two major data sets illustrate spatial variability in the FI and its ability to reflect decadal-scale decline of environmental conditions in the Florida Keys. The changes in dominant taxa reflect changes in the suitability of Keys environments for the symbiotic organisms essential to coral-reef habitats (Figure 1). In 1961, sediment samples were taken along two traverses off Key Largo, Florida. Published reports on these samples (Rose and Lidz, 1977; Lidz and Rose, 1989) provided baseline data for comparison with a limited set of samples taken from the same traverses in 1982, and more extensive sets collected in 1991 and 1992 (Cockey *et al.*, 1996). Mean FI values for samples collected from thriving reef environments in 1961 were 7, with a coefficient of variation (CV) of 0.04 (Table 3). In 1982, the mean FI had plummeted to 4, while the CV had increased to 0.15. By the early 1990s, the mean FI had stabilized at approximately 3, and the CV had returned to < 0.04.

The temporal differences between index values in 1991 and 1992 reflect seasonal differences more than interannual differences. The 1991 samples were taken in September at the end of a climatologically quiet summer and represent peak accumulation of smaller tests of heterotrophic foraminifers. The 1992 samples were taken in May and reflect the effects of higher energy conditions during the winter months. Sites more exposed to storm waves show higher values that reflect those seasonal effects. The 1982 samples are particularly interesting because they reflect a reef ecosystem under stress (e.g., Dustan and Halas, 1987), but not yet in the collapse observed in the 1990s (Jaap *et al.*, 2001). In 1982, shells of smaller foraminifers dominated locally, but in locations more exposed to wave energy, the long-term accumulation of larger foraminiferal shells had not yet been overwhelmed by the more recent production of smaller, more easily sorted shells.

4.3. DISCRIMINATORY ABILITY

Another set of FI values (Figure 2) was calculated from samples collected in 1985 along onshore-offshore transects at La Parguera, Puerto Rico (Donnelly, 1993).

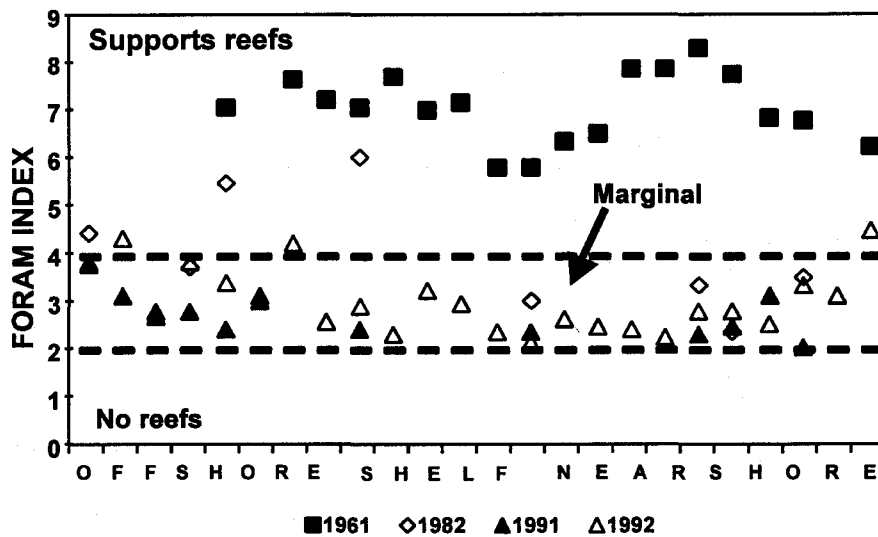


Figure 1. Spatial and temporal variability and interdecadal changes in the FORAM Index for samples from traverses off Key Largo, Florida.

Table 3. Intersample variability in FI values presented in Figures 1 and 2.

Location	Comparison	# Samples	Mean FI	CV*
Key Largo	1961	18	7.09	0.039
Key Largo	1982	8	4.02	0.145
Key Largo	1991	12	2.75	0.038
Key Largo	1992	24	3.07	0.028
Puerto Rico	Inshore	20	2.69	0.026
Puerto Rico	Midshelf	19	3.74	0.197

*Coefficient of Variation

Inshore reefs were in collapse from coastal nutrification and mid-shelf reefs were exhibiting stress (Hallock, 1988). FI values from mid-shelf samples are higher and more variable than nearshore FI values. The shells of smaller foraminifers locally overwhelmed the larger foraminiferal shells that had accumulated over decades of reef growth, although the larger taxa were still common at midshelf sites. On the inner reefs the shells of smaller, fast-growing foraminifers overwhelmingly dominated in the sediments.

An early concern with the FI was that the index might be unduly influenced by sediment grain size. However, plotting indices against median grain size indicates

that, particularly in the most common sediment-size ranges of medium to coarse sands, the FI seems to be relatively unaffected (Figure 3). Intuitively, it is obvious that the shells of smaller foraminifers, as well as juveniles and fragments of larger taxa, should be concentrated in fine sands and muds ($\phi > 3$). However, it is also well known that coral reefs thrive where there is significant water motion, so the presence of muddy sediments can indicate a relatively unfavorable environment, particularly if muddy sediments cover slightly older reef sands and hardbottoms. Hallock (1988) noted that nutrification promotes the production and accumulation of carbonate muds in reef environments for three reasons: (1) Limited nutrification may increase growth and production by calcareous green algae, which break down to mud-size sediments; (2) Nutrification increases rates of bioerosion of reef substrate by endolithic sponges, which etch out mud-size fragments; and (3) Nutrification can promote the growth of algal and bacterial biofilms and mats that baffle and bind finer sediments, allowing those sediments to accumulate. During sampling of inner reefs at La Parguera, Puerto Rico, in 1985 and at Tennessee Reef, Florida Keys, throughout the 1990s, several centimeters of gelatinous carbonate muds overlying sand, rubble and dead-reef substrate in depressions among diseased and heavily bioeroded corals was commonly observed (Pamela Hallock, Personal Communication). The very presence of abundant gelatinous muds in a reef environment indicates a declining environment and will be reflected by a low FI.

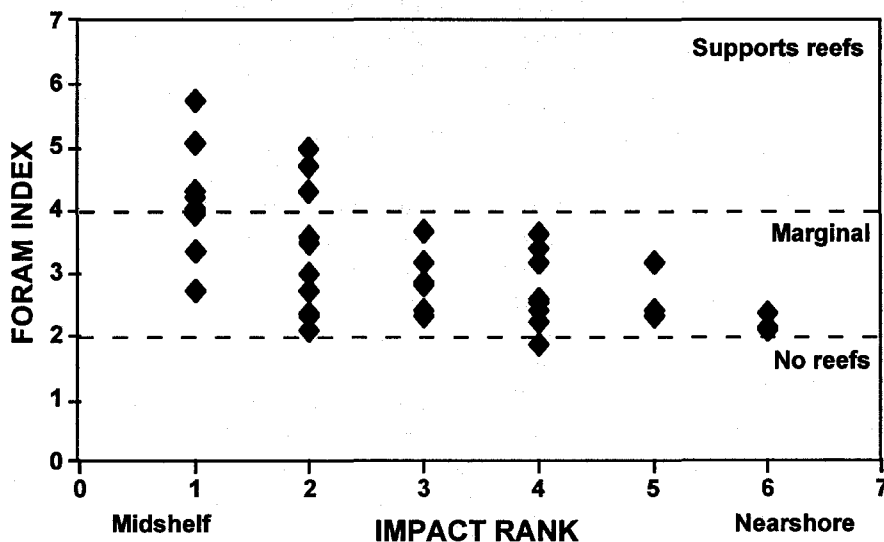


Figure 2. FORAM Index for samples collected from a declining reef system off La Parguera, Puerto Rico.

Equally intuitive is the assumption that coarse grain sizes should be biased toward larger foraminifers. However, the frequency of FI in the 2–4 range (Figure 3) indicates that, where the environment favors the proliferation of smaller foraminifers, they can overwhelm the larger taxa even in samples dominated by coarser sediment sizes, an observation first noted by Cockey *et al.* (1996). Additional testing of the FI on reefs in the Indo-Pacific is needed to resolve whether values from coarse grain sizes are consistently reliable. However, larger foraminifers are important contributors to beach sands of Indo-Pacific reef environments where water quality favors coral growth. Hottinger (Personal Communication) found that nutrification by agricultural runoff to nearshore waters of Mauritius in the Indian Ocean resulted in the loss of *Amphistegina*-sand beaches.

The FI is also independent of sample depth (Figure 4). Incorporating both the shallower-dwelling larger Miliolida (Table 1) and the reef-margin-favoring *Amphistegina* spp. minimizes the potential for depth bias and extends the applicability of the FI from nearshore patch reefs to reef-margin conditions.

5. Interpretative Utility

According to Engle (2000, p. 3–14), “The value of an index lies in its applicability across large geographical areas and its ability to provide regional assessments of ecological condition.” The FORAM Index has been developed using data from Puerto Rico (Caribbean), the Florida Keys (western Atlantic), Hawaii (Pacific),

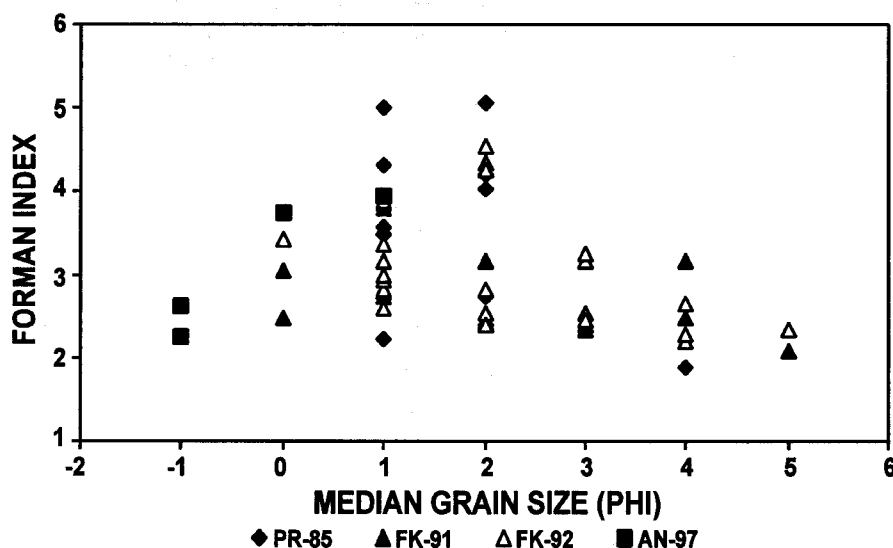


Figure 3. FORAM Index plotted against grain size for reef samples from the Florida Keys (FK), Puerto Rico (PR) and Antigua (AN); year collected is also shown.

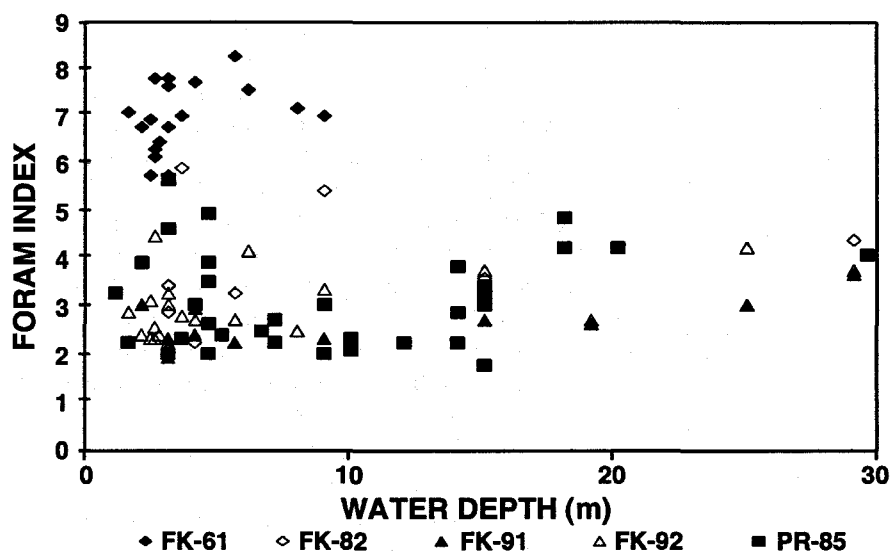


Figure 4. FORAM Index plotted against water depth for reef samples from the Florida Keys (FK) and Puerto Rico (PR); year collected is also shown.

and the Australian Great Barrier Reef (Pacific). Although additional testing in Indo-Pacific reef environments is needed, the FI should have global applicability.

The range of reef-associated habitats that the larger foraminifers occupy indicates that an index weighted for larger foraminifers can be used in both nearshore and offshore reef-associated environments to at least 20-m depth. The larger Miliolida (worldwide, but especially in the western Atlantic and Caribbean), many Calcarinidae and one species of *Amphistegina* (in the Indo-Pacific) are shallow-dwelling taxa and are abundant in nearshore environments where water quality is high. Cockey *et al.* (1996) showed that nearshore larger taxa decline in abundance as nearshore conditions decline and that reef-margin taxa, particularly *Amphistegina* spp., decline as environmental conditions decline at the reef margin (see Figure 1). This diversity of larger foraminifers in reef-associated habitats expands the usefulness of these foraminifers as indicators in reef-associated environments.

6. Discussion

The purpose of EMAP is to provide information on the condition of the Nation's ecological resources (Summers *et al.*, 1995). A new bioindicator is not needed to meet that goal in reef environments; percent live coral cover is probably adequate, with values less than 10% indicative of serious decline (e.g., Dustan and Halas, 1987; Hughes, 1994; EPA, 1998). Most of the Nation's coral reefs, particularly

those in the western Atlantic region (i.e., including the Caribbean and Gulf of Mexico) have dramatically declined over the past 30 years as a result of three major problems: new diseases, coral bleaching, and declining water quality. How interrelated these problems are, i.e., coral bleaching and disease, or disease and water quality, is still not fully known. Certainly coral bleaching is related to anomalously high sea-surface temperatures over the past 25 years, implicating global warming. Synthesis of global climate models and coral-bleaching studies indicates that coral reefs may cease to exist as ecosystems over the next 20 to 50 years (Hoegh-Guldberg, 1999). Ozone depletion and resultant increased intensities of biologically damaging ultraviolet radiation (UV_b) have also increased over the past 25 years and very likely contribute to both temperature stress and susceptibility to disease.

Thus, the major hope for the future of western Atlantic coral reefs as ecosystems is that surviving corals can adapt to temperature stress and disease, and that their descendants can repopulate reef environments. New studies of coral bleaching indicate that surviving corals may exchange lost symbionts for more heat- or light-tolerant strains (e.g., Rowan, 2000; Baker, 2001). In addition, the possibility exists that scientists can genetically engineer heat- and disease-resistant strains of corals and zooxanthellae that can be reintroduced to reef environments. But for either of those optimistic scenarios to be possible, water quality of coastal environments either must be maintained or, where currently contributing to the decline of mixotroph-based communities, must be improved.

Thus, a major question remains unanswered in areas such as the Florida Keys reefs, where the vast majority of coral populations have been lost over the past 25 years and where the reefs will presumably lack significant coral populations for the foreseeable future. How can resource managers recognize whether environments are suitable for coral-reef regrowth if wild populations or genetically engineered corals can adapt to global change? That is one basic question that the FI can address. Larger foraminifers are not dependent upon corals *per se*, but on the high water quality commonly associated with coral reefs.

Many Indo-Pacific reefs were severely damaged by the 1997–98 mass bleaching event (Hoegh-Guldberg, 1999; Wilkinson, 2000). Nevertheless, there are still significant surviving coral populations and, of course, much higher diversities within coral communities (e.g., Veron, 1995). With very high oceanic water quality offshore from reef resources, for example, in Hawaii, Guam, the Mariana Islands, and American Samoa, the prognosis for survival of Pacific reef resources is much better than in the Florida Keys. Thus, the FI can be applied more conventionally to provide an independent assessment of the suitability of local environments for continued reef growth or recovery in the event of a mass mortality event. Further targeted testing of the index in Pacific reefs is needed to confirm and refine it where regional (oceanic) conditions still support reef growth.

7. Summary

Coral reefs are among the most threatened ecosystems worldwide. The FORAM Index provides a metric for determining whether water quality is suitable for mixotroph-based (i.e., algal-symbiotic-dominant) communities. In the western Atlantic region, where disease and bleaching have profoundly damaged coral communities, the FI can be used to assess whether water quality is sufficient to support reef recovery even in the absence of significant coral populations. In the Indo-Pacific, where significant coral communities still exist, the FI can provide a local, independent indicator of environmental suitability for the continuation of reef growth following a mass mortality event resulting from bleaching, a typhoon, or ship grounding.

Acknowledgments

Development and assessment of foraminifers as bioindicators was funded by the U.S. Environmental Protection Agency ORD-STAR-GAD-R825869. Funding contributing to collection of the foraminiferal data sets includes the National Science Foundation EAR-8407781 and OCE-9203278; U.S. Geological Survey 1434-94-A-1185 and 99HQAG0004; and the National Oceanic and Atmospheric Administration National Undersea Research Program Subcontracts No. 9120, 9204.4, 9221, 9322, 9515, 9609, 9703.66 and 9922. Sampling in the Florida Keys was carried out under Florida Department of Natural Resources Permit Numbers 92S-0031, 93S-0156, and 94S-0034; Florida Department of Environmental Protection Permit Numbers 95S-00063, 96S-016, and 00S-016; and Florida Keys National Marine Sanctuary Permit Numbers KLNMS-23-92, FKNMS 09-93, -(UR)-09-94, 07-95, -(UR)-29-96, -006-97, and -2000-011. We thank Drs. D. Griffin, B. Melzian, L. Robbins, T. Smith, and four unidentified reviewers for their helpful comments on the manuscript.

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Ecological risk assessment and testing for endocrine disruption in the aquatic environment

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Abstract

Field observations of endocrine disruption (ED) in fish and other aquatic species have contributed to a global effort to establish test methods for detecting ED effects in wildlife species. For ecological risk assessment, validated tests are needed for Amphibia, fish and aquatic invertebrates, supported by a tiered approach incorporating mechanistic data and exposure characterisation. The potential for extrapolation of ED data from mammalian to aquatic species may be limited, however, due to significant physiological differences in function and regulation of hormone systems in (aquatic) lower vertebrates and invertebrates. Presently, the OECD is considering a tiered approach for ED risk assessment, incorporating a fish 14-day screening assay (Tier 1); fish development and reproduction tests (both Tier 2); and a fish full life-cycle test (Tier 3). For detection of (anti-)oestrogens, the yolk-precursor protein vitellogenin is an ideal biomarker of exposure and functionally equivalent biomarkers are being sought for (anti-)androgens in fish. At the two higher tiers, impacts are assessed in terms of apical endpoints (e.g. development, breeding behaviour and fecundity) and also gonadal histopathology. Validation of these higher tier tests should include comparison of sensitivity of biochemical and apical endpoints to optimise the value of biomarkers for predicting adverse health effects (e.g. impaired reproduction). The specificity of future OECD fish and amphibian test guidelines for endocrine disrupters needs further consideration through inclusion of mechanistic endpoints based on state-of-the-art molecular endocrinology.

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Keywords: Endocrine disrupter testing; Environment risk assessment

1. Introduction

In terms of direct evidence for impacts of chemical endocrine disrupters (EDs) on animal

health, arguably the most convincing examples published to date relate to aquatic organisms. The primary example relates to the masculinising effects in marine molluscs exposed to tributyltin (TBT), with the consequential ecological damage leading to TBT being withdrawn from use in anti-foulant paint. There is also growing evidence of significant feminisation and impaired fertility in freshwater fish populations exposed to steroidal

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oestrogens via effluent discharges. In both cases, field observations of reproductively abnormal aquatic organisms and population impacts have stimulated major research efforts to understand the mechanisms of endocrine disruption in wildlife (reviewed by Vos et al., 2000). Such ecological studies have proven pivotal in the current debate over endocrine disruption in the environment as a whole and the implications for both human and ecosystem health.

Against this background, the Organisation for Economic Co-operation and Development (OECD) is working towards new endocrine disrupter test guidelines for amphibians, birds, fish and invertebrates. This paper provides a brief overview of these activities and underlines the need for basic research in comparative endocrinology and metabolism of OECD aquatic test species. Moreover, the strategy for ED hazard characterisation also needs to integrate with exposure assessment spanning both laboratory and field data (Hutchinson et al., 2000).

2. Challenges in comparative endocrinology and physiology

From an ecotoxicology perspective, the major global concern for aquatic fauna to date centres around four classes of endocrine disruption: namely, (anti-)oestrogens (e.g. natural and synthetic hormones from effluent discharges), (anti-)androgens (e.g. plant derived sterols from pulp mills), steroidogenesis inhibitors (e.g. tributyltin) and thyroid disrupters (e.g. PCBs) (DeVito et al., 1999; Vos et al., 2000). Since over 95% of animal species are invertebrates, it is also important to consider non-vertebrate hormone systems (e.g. ecdysteroid and juvenile hormones) as potential targets for endocrine disruption (DeFur et al., 1999). Given the momentum of ED research and OECD testing using mammals, it is clearly sensible to use data from mammalian models wherever possible to extrapolate to lower vertebrates (e.g. fish and frogs) in order to minimise further testing for ethical reasons. For example, the rodent uterotrophic assay can identify the intrinsic oestrogenic activity of a substance, a property rele-

vant to all vertebrates; however, to assess the significance of oestrogenicity in the context of risks to fish populations requires testing via aquatic exposure (equivalent to simultaneous dermal, inhalation and oral exposure). Therefore, it is important to recognise that a fish or frog is not simply a rat in water, since there are important differences in lower vertebrate physiology and metabolism which preclude the direct use of mammalian data in quantitative ecological risk assessment.

While there is obvious evolutionary conservation among vertebrate hormone systems (e.g. steroid hormone receptor homologies and the common activities of principal hormones such as androgens in male maturation) (Norris, 1996), beyond this there are significant differences in hormone function and regulation between vertebrate taxa. In many cases these differences reflect different reproductive modes, or differences in environmental physiology. For example, while the principal role of prolactin (PRL) in mammals is stimulation of lactation, in fish PRL is involved in osmoregulation and in amphibians plays a role in co-ordinating thyroid-dependent metamorphosis (Norris, 1996). Recently, it has also been reported that the anti-androgenic metabolites of vinclozolin do not appear to bind with the androgen receptor of the fathead minnow (Makynen et al., 2000) as is reported for rodent systems (Kelce et al., 1994).

In terms of metabolism, for example, while methoxychlor metabolism in rodents is sufficient to produce mono- and bis-hydroxylated metabolites which are able to induce a full uterotrophic assay response (Bulger et al., 1985), the weak vitellogenic response in rainbow trout suggests that fish have a limited ability to metabolise methoxychlor resulting in truncation of vitellogenin induction due to systemic toxicity (Thorpe et al., 2000). It is plausible that the converse may occur for oestrogenic parent compounds, however, information on this aspect is lacking given that only a limited number of substances (typically compounds active in mammals) have been compared in both rodent and fish screens to date.

3. Screening for endocrine activity in fish

While recognising the need to minimise use of animals for screening, there is widespread opinion that to avoid false negatives at Tier 1 screening (from mammalian assays alone) there is a need for a fish screening assay to take account of the unique endocrinology and comparative metabolism of oviparous lower vertebrates. As with all screens, such a fish assay must incorporate endocrine specific endpoints (e.g. vitellogenin induction) to incur minimal use of animals at higher tier testing as demonstrated by Purchase (1999). In this context, there is significant interest by OECD experts in a 14-day fish assay (adapted from OECD test guidelines 204 and 215) which is applicable to several fish species. Xenoestrogens have been successfully evaluated in this manner by Thorpe et al. (2000) and on-going research is considering how to expand this assay to detect other types of endocrine disrupter.

4. Higher tier testing with fish for risk assessment

OECD fish expert discussions have led to active consideration of both partial life-cycle tests and the multi-generation fish full life-cycle test for various ecological risk assessment scenarios. For example, where environmental exposures for a pesticide are relatively brief (several days) but overlap with the spawning period for wild fish, a fish reproduction test may be most appropriate. In contrast, with constant annual release of a synthetic hormone such as ethynylestradiol from municipal effluent discharges, a fish full life-cycle test is more relevant to the continuous exposure scenario (Länge et al., 2001). It has therefore been suggested that consideration be given to two new partial life-cycle tests for use in ecological risk assessment: namely, a fish development test and a complementary fish reproduction test (Hutchinson et al., 2000).

The fish development test concept (duration ca. 100 days) is based upon enhancement of the OECD test guideline 210 embryo-larval test and, in principle, could detect chemical impacts on the gross development and growth of fish, whole body

vitellogenin levels, gonadal histopathology and intersex endpoints. This phenotypic abnormality has been widely observed in wild fish populations and also in many laboratory studies of xenoestrogens (reviewed by Vos et al., 2000). The 42-day fish reproduction test is a focal point of US government research efforts and promises to be a highly valuable assay for detecting adverse impacts of endocrine disrupters on pair-breeding adult fish. In principle, such a test could provide valuable data on gross morphology and secondary sexual characteristics, plasma vitellogenin levels, fecundity and gamete quality (Harries et al., 2000).

Finally, in terms of higher tier fish testing for endocrine disrupter risk assessment, the full life-cycle test remains the 'gold standard'. While originally developed by the USEPA in the context of setting water quality criteria for pesticides and other aquatic contaminants, there appears to be significant scope for optimising this complex test to address endocrine disrupters (e.g. inclusion of vitellogenin and gonad histology endpoints) (Länge et al., 2001). In the broad context of higher tier fish testing, the use of selected biomarkers (e.g. vitellogenin, spiggin and gonad histology) is desirable in order to provide laboratory datasets which can be compared with those gathered from wild fish populations. This approach offers a practical means to developing 'linkage tools' for iteration between field and laboratory, being deployed as necessary to support the predictive risk assessment process (Hutchinson et al., 2000).

5. Higher tier testing with amphibians for risk assessment

Based on current evidence of chemical-related thyroid toxicity in several species, there is a strong case for seeking to protect those wildlife species such as amphibians which are arguably exceptionally vulnerable to such chemicals (DeVito et al., 1999). Amphibian metamorphosis is stimulated by thyroid hormones (T_3 and T_4), the synthesis of which is dramatically increased as metamorphic climax is reached. However, metamorphosis is not solely controlled by thyroid hormones and

prolactin is able to inhibit natural (or T_3 -induced) metamorphic changes such as tail resorption (Shi, 1999). Indeed, it appears that increasing prolactin levels approaching metamorphic climax antagonise TH activity at the level of gene expression in order to co-ordinate a sequence of tissue transitions (Tata, 1996). Moreover, both stress and sex hormones are able to influence metamorphosis, and the function of the sex steroid axis in anuran amphibians is controlled by the thyroid axis. Hence, anuran larvae are incapable of synthesising vitellogenin as hepatic expression of the estrogen receptor is induced during metamorphic climax by thyroid receptor (May and Knowland, 1980; Norris, 1996).

In principle, an amphibian metamorphosis assay has considerable potential for detection of thyroid disruptors, as reflected in recent discussions towards the OECD establishing future test guidelines for endocrine disruption in amphibians (first OECD Amphibian Consultation meeting held in April 2001). However, current metamorphosis assays with amphibians are predominantly apical in nature, and have potential to incorporate effects mediated via thyroid and other endocrine disruptors (reviewed by DeVito et al., 1999). Further research is required in order to explore the potential of such an assay, and to develop biochemical or molecular endpoints to provide the necessary specificity to thyroid disruption (i.e. information on mechanism of action). Moreover, it is important that research in this area be conducted in close co-operation with development of thyroid disruption endpoints in mammalian models, in order to establish whether a mammalian screen (incorporating thyroid endpoints) would generate false negatives in relation to lower vertebrate species. Due to the multiplicity of mechanisms through which the thyroid axis can be disrupted, the ultimate role of an amphibian metamorphosis assay in a tiered risk assessment framework for EDs will depend on this taxonomic cross-validation, as well as the relative specificity, sensitivity and reproducibility of thyroid-relevant endpoints in mammalian and amphibian models.

6. Conclusions

Prompted by several reports of endocrine disruption in fish, amphibians and aquatic invertebrates, the establishment of robust ecotoxicity test methods is a high priority for the international scientific and regulatory community. Keeping in mind that ecological risk assessment stands on the two legs of field monitoring and laboratory testing, there is a need to ensure that new OECD test guidelines incorporate priority endpoints (e.g. vitellogenin and gonad histology) which are also used in environmental monitoring programmes. Ultimately, this will be the real-world judgement of laboratory data which are essential to support scientifically meaningful and ethically justifiable endocrine disrupter test guidelines.

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Hallock, P., K. Barnes, and E.M. Fisher. 2004. From satellites to molecules: A multiscale approach to environmental monitoring and risk assessment of coral reefs. *Journal of Environmental Micropaleontology, Microbiology, and Meiobenthology*. 1: (in press).

ABSTRACT

Coral reefs are in decline worldwide. Resource managers require indicators of the biological condition of reef environments that can relate data acquired through remote-sensing, water-quality and benthic-community monitoring to stress responses in reef organisms. A multi-level approach to monitoring and risk assessment of coral-reef resources is proposed that can provide managers with affordable procedures upon which to base management decisions. Advantages of implementing and further developing the proposed approach can be immediate, as well as long term and economic. Most of the technology and procedures are currently available. The ongoing global alert system, based on satellite data, should be expanded to include both estimates of radiant energy reaching the sea surface and water transparency anomalies (i.e., 'photooxidative hotspots' and 'plume' alerts). Satellite-derived alerts should be verified locally by *in situ* physical and chemical measurements, community assessments and bioindicators of stress that can be affordably incorporated into any management plan. Risk assessments based on *in situ* measurements and low cost bioindicators will provide resource managers with essential information to decide when more costly chemical or molecular procedures are needed to determine local sources of stress, which can then focus management actions to protect or restore reef resources.

INTRODUCTION

Coral reefs, along with rainforests, are among the most ecologically diverse ecosystems on Earth. Like tropical rainforests, coral reefs have evolved complex, interdependent community structures (Southwood, 1996; Hill and Hill, 2001) despite, or more likely, because of the paucity of nutrient resources in their environments (Hallock, 2001a,b). A critical factor enhancing diversity in both systems is the abundance of symbiotic and other interdependent relationships that enable tight recycling and efficient use of limited nutrient resources. Another important factor is the vertical structure provided by the most prominent members of these communities (i.e., corals and giant trees), which provides an almost infinite variety of microhabitats for associated organisms (e.g., Southwood, 1996; Hill and Hill, 2001).

In the last three decades of the 20th century, scientists witnessed the decline of coral reefs, first locally, then over whole reef tracts and regions. By the late 1990's, most scientists recognized that coral reefs had declined worldwide (e.g., Dight and Scherl, 1997; Eakin and others, 1997; Risk, 1999; Wilkinson, 1998). Bryant and others (1998) estimated that 58% of the world's coral reefs were threatened by human activities such as sewage and industrial pollution, deforestation, and overfishing. The Bryant report was being released as the 1997-98 ENSO event was triggering mass bleaching of corals unprecedented in its global scale and intensity (Hoegh-Guldberg, 1999). Wilkinson (2000) subsequently estimated that more than a quarter of the world's coral reefs have

been lost, more than half of those to 1997-98 mass bleaching events and their consequences.

Factors contributing to the decline of coral reefs include truly local impacts, local impacts that are so widespread as to be essentially global in scale, regional-scale impacts, and human activities that are altering the Earth's atmosphere, climate, and ocean chemistry. The underlying problem is the ever-growing number of humans on the planet, combined with modern technology. One small piece of the goal to develop sustainable cultures is to reduce anthropogenic impacts on invaluable natural resources such as coral reefs.

THREATS TO CORAL REEFS

The evidence for reef decline ranges from the obvious, such as burial under fluvial sediments or mine tailings, to the gradual replacement of reef-building corals by macroalgae and sponges (e.g., Brown, 1988, 1997; Hallock and others, 1993; Harborne and others, 2001). The causes of burial are generally straightforward and the consequences are typically permanent loss of reef-constructional capability (Hatcher and others, 1989). On the other hand, the causes of gradual changes in community structure continue to be controversial and arguments rage as to whether reef decline is "anthropogenic" or "natural" (e.g., Szmant and Forrester, 1996; Lapointe, 1997; Risk, 1999; Szmant, 2002).

Among the most obvious natural events that impact coral reefs (Table 1) are storms that temporarily reduce salinity while increasing sediment and nutrient loads, i.e., episodic alteration of water quality (Larcombe and others, 2001). As a consequence, the emergence of even small freshwater streams naturally produce breaks in fringing reefs (Torres and others, 2001). Hurricanes, typhoons and cyclones can induce massive physical damage to coral colonies, especially branching species (e.g., Done, 1992; Rogers, 1993; Blanchon and Jones, 1997). Those same branching species are typically fast-growing and recruit from fragments, allowing rapid recovery from even extensive physical impact if environmental conditions are otherwise amenable (Connell, 1997). Biotic interactions such as parasitism, bioerosion, overgrowth, grazing and predation are also natural processes in any ecosystem (e.g., Carpenter, 1997; Glynn, 1997; Peters, 1997). However, these interactions can become serious problems when the ecosystem is subjected to anthropogenic stresses such as nutrification and overfishing (e.g., Hallock and others, 1993; Brown, 1997; Connell, 1997).

Human activities both amplify natural stresses and produce "new" stresses (Table 1) such as human-synthesized chemical pollutants (Peters and others, 1997). Increased flux of nutrients and sediments into reef environments as a consequence of coastal and upland deforestation for agriculture and urbanization (e.g., Harborne and others, 2001) provide examples of anthropogenic amplification of natural stresses, while many of the accompanying chemical pollutants are new stresses. Global climate change can amplify temperature and photic stress, as well as frequency and intensity of tropical and extratropical storms and ENSO events (e.g., Glynn, 1996; Shick and others, 1996; Hallock, 2001a).

THE CHALLENGES

Major challenges for coastal management include recognizing and managing activities that result in new stresses and locally amplified natural stresses, while distinguishing damage from stresses that are being amplified by regional and global changes. Although local managers can do little to reduce either natural stresses or regional and global amplifiers, they can work to minimize local amplification and to ensure that local water quality supports ecosystem resilience.

Risk (1999) assailed the marine-science community for failing the world's coral reefs. He argued that neither lack of monitoring strategies nor lack of monitoring effort were factors in reef decline. Jameson and others (2002) described reef monitoring programs as "ubiquitous". Risk's (1999) primary recommendation was to develop "community-based bioindicator" programs designed to link monitored parameters to management efforts to preserve remaining reef resources.

Jackson and others (2000, p. vii) defined an ecological indicator as "a measure, an index of measures, or a model that characterizes an ecosystem or one of its critical components". They further noted "the primary uses of an indicator are to characterize current status and to track or predict significant change. With a foundation of diagnostic research, an ecological indicator may also be used to identify major ecosystem stress." McCarty and others (2002) noted that the established definition of biomarker (NAS/NRC, 1989) emphasized measurement rather than establishing cause-effect linkages. McCarty and others' (2002) definition of bioindicator emphasizes that it must be "quantifiable" and either "correlated or causally linked to effects at one or more of the organism, population, community or ecosystem levels of organization" (p. 161). McCarty and others (2002) concluded that "Success in adoption of the bioindicator concept in environmental risk assessment will depend largely on improved understanding of the fundamentals of ecosystem structure and function" (p. 163).

To address the challenges of risk assessment in reef systems, reef managers need to choose a toolbox of reliable and economically feasible indicators, including physical, chemical, geological, and biological parameters, which can be used both to assess and monitor coral reef ecosystem structure and function, and to diagnose specific problems. Jameson and others (1998, 2001) made a giant step toward this goal by summarizing attributes and metrics currently available for reef-ecosystem assessment. Risk and others (2001) recommended a bi-level approach to assessment: a) utilizing simple, low technology techniques to detect evidence for stress on reefs anywhere in the world, regardless of local technological resources, and b) the use of physical and geochemical measurements to identify sources of stress. The challenge with any individual or suite of bioindicators is to successfully and economically adapt the procedures to reef assessment, and to understand both their sensitivity and specificity. The U.S. Environmental Protection Agency has developed useful guidelines for evaluating ecological indicators that include conceptual relevance to ecosystem function, feasibility of implementation, response variability in time and space, and ability to convey information on ecological condition that is meaningful to resource management (Jackson and others, 2000).

Since corals are the defining members of coral-reef communities, the obvious question is "Why not use corals as bioindicators?" Coral communities have been monitored for more than two decades (Risk, 1999; Gardner and others, 2003) and strategies for monitoring are numerous. Jameson and others (1998) listed 9 categories and

approximately 40 references for potential scleractinian coral-based bioindicators. Nevertheless, interpretable parameters remain elusive (Brown, 1988; Risk, 1999). Jameson and others (1998) found no formal interpretive framework for any categories except coral fecundity and recruitment, although Ginsburg and others (2001) and Risk and others (2001) have since recommended coral mortality indices as potentially the most useful direct coral-based procedures. Such an index was developed by Gomez and others (1994) and effectively used in Indonesia to distinguish between polluted and non-polluted reefs by Edinger and others (1998). Other parameters can be problematic, in part because corals are long lived and coral community responses to chronic stress may be relatively slow. For example, coral cover can be deceptive because long-lived adults may remain healthy where juveniles can no longer recruit, and conversely, larvae may recruit into areas that cannot sustain reef growth over years to decades (Kinsey, 1988). Diversity in western Atlantic reefs can remain high despite precipitous decline in coral cover (e.g., Porter and others, 2002). Even coral growth rates can be rapid where corals are dying, as described by Edinger and others (2000) and explained physiologically by Hallock (2001b).

A strong case can be made for non-coral bioindicators if they can be demonstrated to effectively reflect coral community function and responses to stress. Ideally the bioindicators should respond rapidly and measurably to specific stresses and not require consumptive sampling of corals or other protected species. Consequently, micro- and meiobiota have great potential as indicators in reef ecosystems (Jameson and others, 1998). Techniques for microbiota, which are already developed for public health applications, should be adapted for use in coral reef ecosystems (e.g., Griffin, 2001; Griffin and others, 2001; Lipp and others, 2002). Like other biota, microbiota are characteristically diverse in reef ecosystems, so lack of taxonomic information can be an impediment. Exceptions are the Foraminifera, for which generic-level taxonomy has been well known for decades (e.g., Loblich and Tappan, 1987).

The goals of this paper are: a) to examine some of the tools that are currently available for risk assessment and monitoring of coral reefs, b) to propose a suite of tools, including micro- and meiobiota, that can be tailored to local resources, c) to discuss how these tools can assist local resource managers in recognizing types and sources of stress, thereby providing evidence to support sound and defensible management decisions; and d) to recommend other promising approaches, including some which utilize micro- and meiobiota, that should be further developed and evaluated to strengthen diagnostic capability.

TOOLS TO ADDRESS SPECIFIC QUESTIONS

ARE CORALS STRESSED?

The first issue to be addressed when assessing a reef ecosystem is the simple question "are the corals stressed?" A number of researchers have suggested that corallivores can provide evidence for stress in corals by either their presence or absence. Outbreaks of Crown-of-Thorns starfish (*Acanthaster planci*) or *Drupella* snails on coral reefs might indicate that corals are stressed and therefore unable to fend off predators (Jameson and others, 1998). On the other hand, Reese (1981, 1994) proposed using obligate corallivorous butterflyfish to indicate coral stress because they decrease in abundance and diversity with decreasing food quality and quantity. Crosby and Reese

(1996) proposed a monitoring protocol to census butterflyfish abundance as an early warning of stress, which was applied by Ohman and others (1998) on reefs in Sri Lanka. Major limitations of this approach are the low sensitivity of response, since both corals and butterfly fish are relatively long lived, and that corallivorous butterflyfish are not ubiquitous in reef ecosystems.

Molecular biomarker technology is a promising approach for identifying and quantifying stress responses in corals and other reef organisms (e.g., Downs and others, 2000; Anderson and others, 2001; Morgan and others, 2001). For example, the Molecular Biomarker System (MBS), introduced by Downs and others (2000), uses the diagnostic tools of biomedical research to assay specific parameters of molecular and cellular physiology that govern organismal health and fitness. The MBS quantifies cellular and physiological condition of the organism, identifies types of stress (e.g., heat stress, oxidative stress, xenobiotic stress), and indicates if defenses have been mounted against a particular stress (e.g., pesticide, heavy metal, PAH) (Downs and others, 2000, 2001). While research aimed at understanding how molecular biomarkers in specific reef organisms are responding to the environment only recently began, measurement of suites of molecular biomarkers on single samples holds promise for determining sources of stress in corals and other organisms (Downs and others, 2000, 2002). Potential limitations include expense and necessity for consumptive sampling, which resource managers may be reluctant to permit without strong justification. In addition, transport of coral samples is restricted by international treaties, which increases the difficulty in applying these tools in developing countries. Nevertheless, molecular biomarkers are potentially the best tools for identifying sources of stress that more affordable techniques detect.

Indicators of coral stress can also be found within the coral surface microlayer (CSM), which includes both coral mucus and microbiotic assemblages. Frias-Lopez and others (2002) observed distinct differences among bacterial assemblages in seawater, and healthy, diseased and dead coral surfaces. Lyons and others (1998) proposed that DNA damage in microorganisms associated with the CSM could be used as an indicator of stress from biologically damaging ultraviolet radiation (UV-B). Technology is available to develop more extensive microbial assays of CSM. An important advantage of this approach is the direct assessment of coral colonies without destructive sampling. Promising methods include molecular screening procedures to characterize microbial assemblages (e.g., Cooney and others, 2002; Frias-Lopez and others, 2002), assessing for specific indicators of stresses such as UV-B damage (Lyons and others, 1998) and toxic metals (Webster and others, 2002), or detecting lysogenic viral activity in response to pollutants (Cochran and others, 1998). McDaniel and others (2001) are developing a prophage induction assay for use in detecting carcinogenic compounds in marine environments that should be adaptable to reef assessment.

DOES TERRESTRIAL INPUT REACH REMOTE REEF ENVIRONMENTS?

The decline of Western Atlantic and Caribbean reefs has been particularly dramatic over the past 30 years (Gartner and others, 2003), in part because reefs have failed to recover from mortality events (Hughes, 1994; Connell, 1997; Ostrander and others, 2000). Oceanic reefs of the Indo-Pacific appear to be much more resilient (Connell, 1997; Wilkinson, 2002). While fringing and other nearshore coral reefs can obviously be directly impacted by terrestrial sediments, pollutants and pathogens (e.g.,

Paul and others, 1995a,b, 1997, 2000; Szmant and Forrester, 1996; Torres and others, 2001), the potential for anthropogenic pollutants to affect offshore reef systems such as barrier reefs and atolls has remained controversial, as have the causes of decline of offshore reefs (e.g., McClanahan and Muthiga, 1998; McClanahan and others, 2001; Szmant, 2002).

Hallock and others (1993) used remote-sensing images to demonstrate that no Caribbean reef was beyond fluvial influence. They illustrated the Mississippi River plume reaching across the Gulf of Mexico to the Dry Tortugas, the Orinoco River plume extending north across the Caribbean to Hispaniola and Puerto Rico, and the Magdalena plume reaching the Nicaraguan Rise (see also Hallock and Elrod, 1988). Hallock and others (1993) further postulated that anthropogenic amplification of fluvial influence has played a role in the decline in Caribbean reefs. Andrefouet and others (2002) subsequently demonstrated that hurricane-enhanced river plumes from Honduran rivers reached the atolls of Belize, which McClanahan and Muthiga (1998) had assumed were too remote for anthropogenic influence.

Interpretations from satellite imagery have been validated by *in situ* data. Jaap (1984) noted that low salinity water occasionally reaches the Florida reef tract following Mississippi River flooding. King and others (2001) found that flood plumes from the Burdekin River of northeastern Australia reach the middle reefs of the Great Barrier Reef tract, which are 60 km or more offshore. Furthermore, most river runoff is profoundly influenced by human activities; Walsh and others (1984) estimated that the nutrient load of a river is directly proportional to the human population in the watershed feeding the river. Furnas and Mitchell (2001) stated "Terrestrial runoff is the largest source of nutrients directly influenced by human activities" (p. 38).

Paul and others (1995, 1997, 2000) and Griffin and others (1999) used bacteriophage tracers to demonstrate that injection of partially treated wastewater into highly porous limestone results in contamination of Florida Keys coastal waters. Viral tracers were recovered from marine surface waters in as little as 10 hr after seeding of waste disposal systems. These studies provided direct evidence for both pathogen and nutrient contamination of coastal environments. Szmant and Forrester (1996), however, argued that nutrient pollution from the coastal zone of the Florida Keys is quickly taken up by nearshore biotic communities and does not reach the reef tract.

Several researchers have detected terrestrial pathogens in coral mucus. Frias-Lopez and others (2002) found bacterial sequences indicating terrestrial origin in coral surfaces infected by black-band disease. Lipp and others (2002) noted that coral mucus may accumulate enteric microorganisms and suggested analyzing mucus to assess risk to humans as well as to coral populations. Basic protocols for assessing sewage influence on reefs can be adapted from techniques used to assess human health risks in coastal waters (e.g., Griffin, 2001; Griffin and others, 2001).

Risk and others (2001) recommended using a geochemical approach to detecting sewage input to reef ecosystems. Changes in ratios of the stable isotopes of nitrogen ($^{15}\text{N}/^{14}\text{N}$) in the tissues of reef organisms can provide evidence for sewage influence (Risk and others, 1993; Sammarco and others, 1999; Heikoop and others, 2000; Yamamuro and others, 2003). However, as Hallock and others (1993) noted, sewage is only one of many sources of nutrients to a reef that can be increased by human activities.

Hallock and others (1993) also postulated that dust, blown by windstorms originating from areas of Africa that have experienced desertification, could also be influencing Caribbean reefs by further contributing to nutrient loading. Satellite imagery has dramatically demonstrated the transport of African dust (e.g., Shinn and others, 2000; Hayes and others, 2001; Griffin and others, 2002). Geochemical analyses of Pleistocene paleosols of islands in the western Atlantic have revealed that Saharan dust is the principal source of clay minerals in otherwise carbonate provinces (Muhs and others, 1990; Herwitz and others, 1996). However, Hayes and others (2001) have shown that climate change in the past 30 years has significantly increased dust transport to the Caribbean basin. The dust is rich in iron, a key growth-limiting micronutrient (e.g., Duce and Tindale, 1991; Martin and others, 1991; Coale and others, 1996). The dust also transports pathogens including *Aspergerillus sydowii*, a terrestrial fungus that infests seafans (*Gorgonia ventalina*) (Smith and others, 1996, 1998; Shinn and others, 2000). Comprehensive studies of microbes both in dust samples and in the reef environment (Smith and others, 1996, 1998; Griffin and others, 2002) have provided the key to interpreting this mechanism of influence. Hayes and others (2001) also suggested that biologically-available iron in the dust promotes outbreaks of otherwise iron-limited marine pathogens and opportunistic toxic algae.

DOES WATER QUALITY SUPPORT REEF GROWTH?

Coral reefs worldwide have exhibited profound changes in benthic community structure and function over the past 30 years. In many areas, coral or mixed coral-algal communities have been replaced by fleshy and filamentous algae (Dustan and Halas, 1987; Hughes, 1994; Bell and Elmerti, 1995; LaPointe and others, 2002; many others). Phytoplankton concentrations in the vicinity of the Low Isles in the Great Barrier Reef lagoon have significantly increased over the past 65 years, while plankton assemblages have changed from mixed centric and pennate diatoms to predominantly pennate diatoms and small flagellates, indicating community response to anthropogenic nutrients (Bell and Elmerti, 1995).

As nutrient supplies and plankton populations increase, heterotrophic macroinvertebrates, including sponges, tunicates, and barnacles increase in abundance (e.g., Rose and Risk, 1975; Tomascik and Sander, 1987; Kinsey, 1988). Percent cover or abundances of selected heterotrophic macroinvertebrates are included in several reef-monitoring protocols (Hodgson, 1999; McManus and others, 1997; Porter and others, 2002). Jameson and others (2001) considered assessment of both sessile epibenthos and benthic macroinvertebrates to be essential for creating an effective Index of Biological Integrity for coral reefs. Studies in both the Caribbean and Pacific (e.g., Rose and Risk, 1985; Sammarco and Risk, 1990; Holmes, 1997) have demonstrated that internal bioerosion of reef rubble and live coral colonies increases with increasing nutrient flux. Risk and others (2001) proposed a bioerosion index based on work by Holmes (1997) and Holmes and others (2000) on bioerosion in coral rubble.

Erdmann and Caldwell (1997) recommended using stomatopod crustaceans (mantis shrimp) as bioindicators of pollution stress because they are sensitive to a variety of pollutants, they are abundant and ubiquitous on reefs worldwide, they are easily sampled, and non-specialists can readily be taught to identify them. Jackson and others (1989) and Steger and Caldwell (1993) found that these invertebrates are highly sensitive

to oil pollution. Risk and others (2001) concluded that these crustaceans are among the most promising first-level indicators of pollution stress.

The effects of environmental and anthropogenic stresses on amphipod crustaceans have been studied since the 1970's, revealing that these organisms are both behaviorally and physiologically sensitive to a variety of pollutants and toxicants (Thomas, 1993). The U.S. Environmental Protection Agency already uses these crustaceans in marine biomonitoring protocols (e.g., Engle and Summers, 1999). The exceptional sensitivity exhibited by these invertebrates enhances their potential for use as indicators of early stages of stress, permitting time for mitigation or other management decisions. Realization of the potential of this group requires either taxonomic inventories in the regions where they are to be used (Thomas, 1993) or development of more economical molecular-genetic screening protocols (e.g., Rohwer and others, 2001, 2002; Peterson and others, 2002).

MacKenzie (1999) suggested that parasites in marine organisms are a promising indicator of the effects of pollution. Since the free-living transmission stages of such internal parasites as helminthes are highly sensitive to environmental conditions, the incidence of helminth endoparasites tends to decrease as water quality declines. On the other hand, marine ectoparasites tend to be highly tolerant of pollutants, thereby tending to increase in abundance as water quality declines. Jameson and others (1998) noted that timed, visual censuses of fish ectoparasites within areas of concern have been proposed as an assessment tool, though no formal interpretive framework has been developed. Diamant and others (1999) combined parasitological investigations with physiological assessments of rabbitfish in the Red Sea and the Mediterranean, to further demonstrate the potential for using fish parasites as bioindicators.

There is little argument that benthic community structure has changed throughout the Caribbean region over the last several decades. Hallock and others (1993), Lapointe (1997), and Lapointe and others (2002) postulated that anthropogenic amplification of nutrient flux has played a major role in the phase shift to algal dominance of former coral reefs. Numerous other researchers, including Hughes (1994), Miller and others (1999), McClanahan and others (2001) and Szmant (2002) have argued that reduction in herbivory, associated with both overfishing and the 1983 mass dieoff of *Diadema* urchins (Lessios and others, 1984; Lessios, 1988), has enabled macroalgae to take over benthic habitat when corals are killed by other mechanisms. Most monitoring and assessment programs reviewed by Jameson and others (1998, 2001) included sessile epibenthos, macrophytes, benthic macroinvertebrates, and fish in their procedures, most of which Jameson and others (2001) considered to be "non-diagnostic".

Cockey and others (1996) postulated that foraminiferal assemblages in sediments should be useful in determining if changes in water quality have played a role in the decline of Florida Keys reefs. Foraminiferal assemblages include both mixotrophic taxa that are dependent upon algal endosymbionts for enhanced growth and calcification, and purely heterotrophic taxa that are herbivorous and detritivorous. These investigators argued that, if the decline in coral cover in the Florida Keys was the consequence of physical damage, decline in grazing following the *Diadema* dieoff and coral-specific diseases, the foraminiferal assemblages should show little or no change. Cockey and others (1996) demonstrated that foraminiferal assemblages in the sediments profoundly changed in Florida reef-tract sediments, from domination by algal-symbiont bearing

larger taxa in the 1960s to domination by the smaller heterotrophic taxa in the 1990s. This shift in the foraminiferal assemblage is ecologically consistent with the decline in reef-building corals and their replacement by benthic algae and sponges over the same period. Cockey and others (1996) concluded that increasing nutrient flux to the reef tract was likely impacting community structure.

Based on data sets collected by Cockey and others (1996) in the Florida Keys, Donnelly (1993) in Puerto Rico, and Hammond and others (2000) in Antigua, Hallock and others (2003) proposed and evaluated a single-metric index based on foraminiferal assemblages from sediment samples using guidelines proposed by Jackson and others (2000). The resulting Foraminifera in Reef Assessment and Monitoring (FORAM) Index provides a tool for assessing whether water quality in the environment is adequate to support mixotrophy (i.e., algal symbiosis) as the dominant nutritional mode in an ecosystem. The FORAM Index can also be used for risk assessment of proposed restoration projects, as well as assessment of management outcomes. A key advantage of this index is that it is based on functional groups of Foraminifera, which are recognizable worldwide, thus minimizing the need for local taxonomic specialists.

Lidz and others (1985) and Hallock (1988) suggested that the composition of reef sediments as a whole also reflects benthic community structure and function. The basic premise is that mixotrophic organisms, particularly those that calcify, are more successful competitors in low nutrient environments. As a consequence, reef sediments can be dominated by readily identifiable skeletal components, particularly larger foraminifers (McKee and others, 1956). As nutrient supplies increase, skeletal fragments of calcareous algae and herbivorous gastropods increase in abundance, as do bioeroded coral fragments. When nutrient supplies increase even further, such that fleshy algae, boring sponges and bivalves become increasingly prevalent, recognizable coral and larger foraminiferal constituents become increasingly rare, while unrecognizable fragments, along with the shells and fragments of heterotrophic foraminifers and bivalves, become dominant. Because carbonate sediment composition reflects community structure and function, this technique also can be applied as an assessment tool worldwide. Lidz and Hallock (2000) used this model to interpret changes in sediment constituents along the Florida reef tract. Further evaluation is needed to produce a standardized protocol.

IDENTIFYING SPECIFIC STRESSES

Specific stresses that human activities are amplifying include extremes of temperature, irradiance, salinity, oxygen, nutrients, trace elements, and sedimentation. New anthropogenic stresses include synthetic hydrocarbons such as pesticides and herbicides, artificial radionuclides, antibiotics and others. In the continuum between natural, amplified-natural, and new stresses are the nearly infinite variety of hydrocarbons ranging from crude oil to combustion products. Unfortunately, stresses are seldom singular. The most common impact on water quality, increased nutrient flux associated with agricultural runoff and sewage, is typically associated with increased sediment flux, along with a brew of pesticides, herbicides, fungicides, antibiotics, and hydrocarbons.

Specific effects of most chemicals on marine organisms are poorly known. Richmond (1993) demonstrated that selected organophosphates can inhibit settlement of

coral larvae and McKenna and others (2001) developed procedures for utilizing this information in assessment. However, since corals generally spawn annually, availability of larvae restricts the widespread applicability of such methods.

Several efforts to detect gene expression to specific pollutants have been reported. Ammar and others (2000) assayed expression of the metabolic enzymes fructose-1,6-bisphosphatase and succinate-dehydrogenase in an azooxanthellate octocoral by isolating the cDNA for both. The levels of expression of these enzymes were correlated with stress from sedimentation. Morgan and others (2001) presented a method for utilizing gene probes as toxicant-specific biomarkers of coral stress responses. Morgan and Snell (2002) used this technology to determine exposure of the coral *Acropora cervicornis* to dibrom, a pesticide used in mosquito control in south Florida.

The MBS (Downs and other, 2000) assays for general indicators of cell integrity including glutathione and ubiquitin, molecular chaperones that indicate the integrity of enzyme pathways, small heat-shock proteins, and antioxidant enzymes indicative of oxidative stress. Examining an array of stress responses provides the potential to differentiate between a global stressor (e.g., high sea-surface temperature) and a local stressor (e.g., fungicide, an organometalloid, endosulfan) (Downs and others, 2000; 2001; 2002). The MBS also is able to differentiate between bleaching due to oxidative stress and bleaching due to other stresses because cells respond differently (Downs and others, 2002).

Peters and others (1997) examined three classes of pollutants on reefs, heavy metals, petroleum, and synthetic organics including herbicides and pesticides. They concluded that heavy metal pollution has caused physiological stress, reduced reproductive success and mortality in fish and invertebrates, though risk assessment criteria are needed. Haynes and others (2002) reported that Great Barrier Reef management is focusing on heavy metals, persistent chlorohydrocarbons, PCBs and petroleum-related compounds originating from agricultural runoff. Owen and others (2002) demonstrated experimentally that measured concentrations of the antifouling *s*-triazine herbicide in coastal waters of Bermuda, Florida Keys and St. Croix in the U.S. Virgin Islands were sometimes sufficiently high to suppress photosynthesis by coral zooxanthellae. Scott and others (2002) found evidence of pesticide runoff, particularly of endosulfan, in south Florida estuaries. The most widely applied biocriterion for pollution is the global "Mussel Watch" program, which monitors incorporation by bivalves of trace metals, artificial radionuclides, petroleum compounds and halogenated hydrocarbons (e.g., Goldberg and others, 1978; Hungspreugs and Yuangthong, 1984).

The antifouling agent, tributyltin, induces imposex, the imposition of male sexual organs on female, in marine snails. Ellis and Pattisina (1990) recognized the potential of this phenomenon as a bioindicator, proposing the "relative penis size index" (RPS Index) to measure the proportional responses of gastropod imposex to tributyltin exposure. Foale (1993) evaluated the index, which is now widely applied and intercalibrated among laboratories (e.g., Davies and Minchin, 2002) worldwide. In tropical and subtropical waters, the RPS Index has been used in monitoring (e.g., Bech, 2002a,b), in assessing the effectiveness of regulations (e.g., Gibson and Wilson, 2003), and in risk assessment using transplant experiments (e.g., Bech and others, 2002).

Yanko and others (1994, 1998), Samir (2000), and Samir and El-Din (2001) studied the responses of benthic foraminifers to a variety of pollutants in the eastern

Mediterranean, including hydrocarbons and heavy metals. These researchers detected not only assemblage responses, but also noted that shells in polluted environments were commonly stunted, pyritized or deformed. The FORAM Index (Hallock and others, 2003) is based upon relative abundances of three functional groups of foraminifers: larger taxa that host algal symbionts, smaller taxa that bloom when nutrification increases food resources, and stress-tolerant taxa that dominate when water quality is seriously impacted by eutrophication, heavy metals, or other pollutants.

Jackson and others (1989) and Steger and Caldwell (1993) studied the effects of a major oil spill on coral reef and associated mangrove communities in Panama, and reported that mantis shrimp were particularly sensitive to oil pollution. Gesteira and Dauvin (2000) concluded that amphipods are useful indicators of the impact of oil spills on soft-bottom communities, noting that polychaete/amphipod ratios can indicate impact or recovery of the soft-bottom macrobenthos. Similarly, assessment of nematod/copepod ratios (e.g., Ansari and Ingole, 2002; Montagna and others, 2002) can indicate impact or recovery of meiobenthos following a spill.

Solar radiation reaching the sea surface and the seafloor are also natural parameters that are being anthropogenically modified. A major factor in coral bleaching is photooxidative stress; elevated water temperatures lower the threshold for photoinhibitory damage to Photosystem II in coral zooxanthellae (Lesser, 1996, 1997; Warner and others, 1999; Downs and others, 2002; others). Stratospheric ozone depletion has progressed to the degree that biologically damaging UV-B intensities previously experienced only near the summer solstice on mid-latitude reefs are now experienced from April through August (Shick and others, 1996). Thus, at the same time that global warming is producing longer, warmer summers, particularly during ENSO years, UV-B reaching the sea surface has also increased. Furthermore, mass bleaching events in 1983, 1987-88 and 1991 (Hoegh-Guldberg, 1999) followed major volcanic eruptions that depleted stratospheric ozone globally by as much as 4% (Randell and others, 1995; Roscoe, 2001). UV-B penetrates highly transparent oceanic waters sufficiently to have both direct and indirect effects. Gleason and Wellington (1995) found UV-B damage in corals and coral larvae. Lyons and others (1998) documented UV-B induced DNA damage in microorganisms found in the coral-surface microlayer.

Bleaching in both zooxanthellate corals (Fitt and Warner, 1995) and reef-dwelling foraminifers (Hallock and others, 1995; Williams, 2002) has been shown experimentally to be accelerated by exposure to shorter, higher energy wavelengths of solar radiation, including the violet to blue (400-500nm), UV-A (320-400 nm), and UV-B (280-320 nm). Anderson and others (2001) showed that warm, calm, stratified sea surface waters promotes photobleaching of chromophoric dissolved organic matter (CDOM), further enhancing penetration of shorter wavelengths of solar radiation. Williams (2002) documented that bleaching intensity and prevalence in the foraminifer *Amphistegina gibbosa* in the Florida Keys peaks with the summer solstice, rather than with the late summer thermal maximum. She also demonstrated, using SEAWIFS satellite imagery, that prevalence and intensity of bleaching in *A. gibbosa* was inversely related to CDOM concentrations in overlying waters. Toler (2002) found that shell damage by predation or infestation in these foraminifers was most prevalent at intermediate bleaching incidences, indicating that the stress that induces partial bleaching can reduce the foraminifers' ability to avoid predation and infestation.

A BASIC TOOLBOX

Tools that are currently available to assess and monitor stresses and impacts on coral reefs are numerous (e.g., Jameson and others 1998, 2001) and range from satellite imagery to microbial and molecular assays. Potential tools based on state-of-the-art technology are also plentiful. This section examines several basic classes of tools and suggests ways to expand their usefulness.

REMOTE SENSING

Satellite-derived sea surface temperatures (SSTs) are currently being used to predict coral mass-bleaching events (Gleeson and Strong, 1995; Carriquiry and others, 2001; Aronson and others, 2002). Because coral bleaching events have been noted in areas where the SST exceeds the climatological maximum for that region by 1° C or more, charts that illustrate anomalies are updated at least weekly (NOAA/NESDIS Coral Reef Team, 2003).

Satellite technology is in place that could support similar monitoring of total ultraviolet and visible solar radiation reaching the sea surface and the sea floor at shallow-reef depths. For example, Mumby and others (2001) concluded that dense cloud cover in the Society Islands during the 1998 ENSO event limited bleaching damage despite high water temperatures. A photic-stress index could be developed that is based on UV-B, UV-A, and visible (400-700 nm) radiation reaching the sea surface, which can be estimated based on stratospheric ozone, cloud cover, and aerosols. Algorithms that remove the effects of clouds and aerosols and that distinguish the effects of water turbidity, chlorophyll pigments and CDOM (e.g., Lee and others, 1998, 2002; D'Sa and others, 2002; Lee and Carder, 2002) can provide estimates of irradiance reaching depth (e.g., 10-20 m).

Irradiance estimates, combined with temperature, would allow prediction of the total photooxidative stress to the benthic community. This same technology would permit tracking ocean color or turbidity plumes that indicate major terrestrial runoff events (e.g., Andrefouet and others, 2002), offbank transport of sediments, dense water plumes (Acker and others, 2002), or other anomalous events (e.g., Hu and others, submitted), which could have detrimental impacts on reefs. Ongoing "coral reef hotspot" monitoring now can direct researchers to where corals can be expected to bleach. Similarly, more comprehensive monitoring that included solar irradiance and ocean color would enable researchers to more effectively determine the interplay between temperature, sunlight and water transparency in their effects on reef communities.

IN SITU MONITORING OF PHYSICAL AND CHEMICAL PARAMETERS

A basic, low cost, low technology monitoring program based at marine laboratories around the Caribbean regularly records selected physical and biological parameters and monitors for change, with the goal of distinguishing natural from anthropogenic disturbance (e.g., Alcolado and others, 2001). A more comprehensive program of reef-based monitoring buoys and regular *in situ* sampling of physical and chemical parameters including temperature, conductivity, wind speed, turbidity, solar radiation, nutrients (ammonium, nitrate, orthophosphate, total N and total P), and chlorophyll concentrations, as has been initiated for the Florida reef tract (e.g., Ogden

and others, 1994; Szmant and Forrester, 1996; Boyer and Jones, 2001). Data from this network can provide measurements needed to calibrate satellite-derived data and establish local stress alerts (e.g., Hendee and others, 2001). Underwater irradiance, turbidity, and chemical measurements tend to be compromised by fouling of the sensors (Nittis and others, 2001), which can take place within hours to days of emplacement. Technology to protect sensors has been recently developed (Williams, 2002). Nevertheless, how to relate the physical and chemical environment to individual, population and community responses remains a major question (Risk, 1999).

COMMUNITY STRUCTURE AND FUNCTION

Taxonomic groups fundamental to reef condition are algae, corals and fish. A variety of survey protocols have been developed, based on objectives and available resources (reviewed by Jameson and others, 1998, 2001). There are volunteer-focused programs such as ReefBase Aquanaut (McManus and others, 1997) and ReefCheck (Hodgson, 1999). The Florida Keys National Marine Sanctuary Coral Reef Monitoring Project monitors coral cover and other parameters at fixed sites (e.g., Porter and others, 2002). Aronson and others (1994) proposed a quick and inexpensive procedure for reef resource assessment. The Atlantic and Gulf Rapid Reef Assessment AGRRA protocol (Ginsburg and others, 2000) has been adapted Caribbean-wide in recent years, and includes coral cover, coral mortality and algal and fish biomass. Miller and others (2002) developed a procedure to assess how benthic habitats respond to protection from fishing. Hudson and Goodwin (2001) provided protocols for assessment following ship groundings on coral reefs. Thus, as Risk (1999) argued, the issue is not how to monitor what is happening to reefs, but rather how to determine causes of change.

Whether a reef community has the resilience to recover from impact may not be reflected by coral measures such as percent cover, diversity or growth rates, but rather by bioerosion rates and whether local water quality supports nutrition based on algal symbiosis. Thus, in areas where coral communities are largely intact, assessments based on stomatopod abundance and diversity (Erdmann and Caldwell, 1997), the FORAM Index (Hallock and others, 2003), sediment constituents (Lidz and Hallock, 2000) or bioerosion (Risk and others, 2001) can indicate if the system is likely to recover if a mass mortality event occurs. Sediments dominated by larger foraminifers and easily recognizable coral constituents indicate that the environment supports algal symbiosis as the dominant modes of nutrition and carbonate production. On the other hand, sediments dominated by algae and molluscan fragments, or difficult to recognize bioeroded and coated grains, indicate that the environment favors autotrophy and bioerosion as dominant processes. In the latter case, when corals are lost to disease or a mortality event, the reef community has a low probability for recovery, particularly if herbivores are scarce as a result of disease or chronic overfishing.

In the western Atlantic and Caribbean, coral cover has been lost to hurricanes (Hughes, 1994), disease (Gladfelter, 1982; Goreau and others, 1998; Ostrander and others, 2000), sedimentation, (e.g., Nemeth and Nowlis, 2001, Torres and others, 2001), oil spills (Jackson and others, 1989), ship groundings (e.g., Gittings and others, 1988), and other events or chronic stresses. Coral cover, which was often in excess of 50% in the 1960s and 1970s, is now commonly below 10% (e.g., Dustan and Halas, 1987; Hughes, 1994; Ostrander and others, 2000; Gardner and others, 2003). In such cases, the

FORAM Index and sediment constituents should be useful in determining if water quality will still support reef growth, even though regional or global stresses (e.g., bleaching, dust-borne diseases, chronic photooxidative stress increasing susceptibility to disease, bleaching) have reduced coral cover. Accompanying fish and urchin surveys can provide evidence as to whether reduced grazing has contributed to algal overgrowth of the substrate (e.g., Miller and others, 1999).

LOCAL VERSUS GLOBAL STRESS: A LOW COST BIOINDICATOR

Where decline has occurred as visually indicated by degrading reef structure or historic records, or is occurring as indicated by coral mortality indices (Gomez and others, 1994; Edinger and others, 2000) or a disease outbreak (e.g., Santavy and others, 2001), the challenge is to determine whether causes are local or global. A promising low cost tool is the response of *Amphistegina* populations (Hallock-Muller, 1996; Hallock, 2000; Williams, 2002).

Amphistegina are commonly the most abundant and one of the most widely distributed, symbiont-bearing foraminifers on reefs and carbonate shelves worldwide (Langer and Hottinger, 2000). Two closely related species, *A. gibbosa* in the western Atlantic/Caribbean and *A. lessonii* in the Indo-Pacific, are known to be sensitive to water quality and to bleach in response to excess radiant energy (Hallock and others, 1986, 1995; Talge, 2002; Williams, 2002). Low-level bleaching stress increases the susceptibility of these protists to predation and infestation (Hallock and Talge, 1994; Toler and Hallock, 1998; Toler, 2002). Richardson and others (2001) recommended assessing bleaching and disease in coral populations in late spring, when disease activity starts to increase, and late summer, when bleaching and disease tend to be highest. These are also the optimum times to assess *Amphistegina* populations to determine if water quality is favorable for mixotrophic nutrition, based on abundance (Hallock-Muller, 1996; Hallock, 2000), or if photic stress is likely to induce susceptibility to disease or bleaching in mixotrophs, based on size distribution, bleaching prevalence and intensity (Williams, 2002), or breakage prevalence (Toler, 2002).

Responses of *Amphistegina* populations can provide managers worldwide with a relatively quick, low cost method to make informed decisions about when to employ more expensive technology, such as the $\delta^{15}\text{N}$ assessment (Risk and others, 2001), the MBS (Downs and others, 2000, 2002) or other specific detection protocols to determine sources of stress, including DNA damage (Anderson and others, 2001), heat stress (Weins and others, 2000), or pesticides (e.g., Morgan and Snell, 2002). A key advantage of regular monitoring of *Amphistegina* is their visually quantifiable and distinct responses to water quality and chronic photooxidative stress.

OTHER POTENTIAL TOOLS IN THE MICRO- AND MEIOBIOTA

Other micro- and meiobiota provide the potential for development of additional tools for reef monitoring and risk assessment. In particular, changes in the microbiota in the CSM is the logical place to look for clues to disease susceptibility. Richardson and others (2001), Ritchie and others (2001), and Santavy and others (2001) have proposed strategies and methodologies for assessing and investigating disease incidences and outbreaks. As noted previously, several researchers have detected terrestrial pathogens in corals or their mucus (Lipp and others, 2002; Patterson and others, 2002). Promising

methods include molecular screening procedures to characterize microbial assemblages (e.g., Cooney and others, 2002; Frias-Lopez and others, 2002), assessing for specific indicators of stress (e.g., Lyons and others, 1998; Webster and others, 2002), or detecting lysogenic viral activity in response to pollutants (Cochran and others, 1998; McDaniel and others, 2001). The challenge, as with any bioindicator, is to successfully and economically adapt the procedures to reef research and to understand both their sensitivity and specificity.

Any procedure that has been developed for use in monitoring or risk assessment in other marine environments has the potential to be adapted for use in marine environments. Methods based on taxonomy of micro- or meiobiotic assemblages must confront problems of diverse, poorly known assemblages. However, just as technology is available to assay coral surface microlayer assemblages, similarly assays of sediment microbiota and meiobiota can be used to test for change in assemblages or for specific gene expression in response to pollutants or other stresses.

Amphistegina spp. have other potential applications in risk assessment: laboratory bioassays of water quality and field transplant experiments to determine how they respond to sites or environmental conditions of concern. These foraminifers are large enough to be individually assessed and manipulated, yet they are easily and inexpensively maintained in culture (Hallock, 2000). Research aimed at developing protocols to determine gene expression associated with specific stress responses are critical to expanding application of these protists in reef research.

RECOMMENDATIONS

Potential tools for coral reef monitoring and risk assessment are numerous, though relatively few have been formally evaluated for conceptual relevance, feasibility of implementation, response variability, sensitivity and limitations (see Jackson and others, 2000). The following approach, which includes currently available procedures and technology, as well as recommendations for further development and evaluation, can provide reef managers with solid information upon which to make management decisions (Figure 1):

a) Remote sensing: Currently, global temperature “hot spot” anomalies are posted on a website at least once per week (NOAA/NESDIS Coral Reef Team, 2003). The effort has successfully predicted episodes of mass bleaching in corals (e.g., Carriquiry and others, 2001). Expanding the products of this website to include estimates of radiant energy (visible and UV) reaching the sea surface, as well as seawater absorption coefficients (e.g., Lee and others, 1998; D’Sa and others, 2002; Lee and Carder, 2002), would enable identification of both “photooxidative hotspots” and anomalous absorption or reflection features such as the “blackwater” event off Florida in 2002 (Hu and others, 2002) or river flood plumes (Andrefouet and others, 2002).

b) *In situ* physical and chemical data: *In situ* monitoring should be carried out at whatever level is economically feasible, i.e., ranging from daily measurements by a marine lab staff (e.g., Alcolado and others, 2001) to continuous or hourly data streams from well maintained moored buoys that can support local alert systems (e.g., Hendee and others, 2001). Including UV-A and UV-B sensors, as well as collection of temperature and all irradiance parameters at standard depths (e.g., 10 m and 20 m), would substantially enhance modeling of photooxidative stress at the sea floor.

c) Assessment of community structure and function: A variety of procedures have been developed to assess coral and fish communities, ranging from the volunteer-focused efforts (e.g., McManus and others, 1997; Hodgson, 1999), to protocols that require professional field and laboratory teams (e.g., Ginsburg and others, 2000; Porter and others, 2001; Miller and others, 2002). Sampling of reef rubble and sediments, either during other monitoring efforts or as an independent effort, for assessment of bioerosion (Risk and others, 2001), the FORAM Index (Hallock and others, 2003), or sediment constituents (Lidz and Hallock, 2000), can provide evidence on benthic community function relative to algal symbiosis, calcium carbonate production and bioerosion. These assessments should be carried out every three to five years to assess resources, identify reefs in decline, and focus protection efforts.

d) Low-cost bioindicators of stress: Stomatopod diversity and abundance (Erdmann and Caldwell, 1997; Risk and others, 2001) can be monitored annually for pollution stress. Bleaching, disease and mortality in coral (e.g., Santavy and others, 2001; Risk and others, 2002) and *Amphistegina* populations (Hallock 2000; Williams 2002) should be monitored at sites of interest twice yearly, in late spring and late summer. In addition, if remote sensing, *in situ* physical/chemical monitoring, or community assessment detect evidence or potential for stress, or when a disease outbreak or mortality event is reported, *Amphistegina* populations can provide evidence to distinguish between water quality and acute or chronic photooxidative stress as possible sources.

e) Determining causes of stress: When coral, stomatopod or *Amphistegina* populations indicate stress responses, resource managers are equipped to request or defend decisions to expend resources to identify sources of stress. If sewage pollution is suspected, nitrogen isotope analysis (Risk and others, 1993, 2001; Heikoop and others, 1998, 2001), analysis of CSM biota for sewage-derived microbes (e.g., Frias-Lopez and others, 2002), and viral tracers (e.g., Griffin and others, 1999; Donaldson and others, 2002) can be employed to verify sources and pathways of dispersal. If photooxidative stress is suspected, with or without temperature stress, species should be assessed using the MBS (Downs and others, 2000, 2002) or for DNA damage (Lyons 1998; Anderson and others, 2001). If trace metals or pesticides are suspected, the MBS or gene expression technology (e.g., Morgan and others, 2001) can be employed. While a suite of techniques is currently available for the study of coral diseases (e.g., Richardson and others, 2001; Ritchie and others, 2001; Santavy and others, 2001), additional research and development are needed to optimally utilize the CSM microbiota to diagnose specific stresses and disease outbreaks.

Advantages of implementing and further developing this multi-level approach for risk assessment and monitoring of coral reefs can be immediate, as well as long term and economic. While a suite of tools are currently available to managers to focus actions to protect valuable reef resources, many of the proposed bioindicators and biomarkers require further development and assessment (e.g., Jackson and others, 2000). Marine protected areas, at least in some cases, can provide reference sites for comparison with impacted sites and to aid in distinguishing local impacts from regional or global stresses. The larger challenges are to find the political will and monetary resources to fundamentally improve agricultural, industrial, urban and coastal-development practices

in ways that reduce harmful emissions, runoff, and release of toxic chemicals to the atmosphere and biosphere.

ACKNOWLEDGMENTS

This research was supported by the U.S. Environmental Protection Agency Office of Research and Development's National Center for Environmental Research "Science to Achieve Results Program" (STAR-GAD-R825869), by a subcontract from the Medical University of South Carolina, funded by South Carolina Sea Grant, Dr. Cheryl Woodley and Dr. Eric Lacy, Principal Investigators; by the National Oceanic and Atmospheric Administration/National Undersea Research Center UNCW (9703-66 and 9922); and by USF/USGS Cooperative Research agreements (2000-2003). The opinions expressed in the manuscript are those of the authors; acknowledgment of funding does not imply endorsement of those views by the funding agencies.

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Table 1. Examples of natural stresses, natural stresses that are amplified by human activities, and new anthropogenic stresses that affect coral-reef ecosystems.

- A. Natural processes that can induce stress (examples):
 - Storms (physical damage, sedimentation, reduced salinity, nutrients)
 - Currents (tidal currents or upwelling changing water temperatures)
 - Terrestrial runoff (sedimentation, reduced salinity, nutrients)
 - Oxygen (oxidative stress, insufficient oxygen)
 - Temperature (heat or cold stress)
 - Sunlight (photoinhibition, shading)
 - Biotic interactions (predation, competition, bioerosion, parasitism, disease)
 - Chemical stress (naturally occurring trace metals and hydrocarbons)
- B. Natural stresses that can be amplified by anthropogenic activities (examples):
 - Physical damage (ship grounding, anchor damage, dredging, fishing with explosives)
 - Currents (engineered water flow, changes in salinity or water quality)
 - Terrestrial runoff (deforestation, agriculture, urbanization)
 - Temperature (thermal effluent, circulation changes, climate change)
 - Sunlight (ozone depletion/increased UV-B, reduced water transparency)
 - Biotic interactions (invasive species, overfishing, phase shifts)
 - Chemical stresses (pollution by trace metals, hydrocarbons)
- C. New stresses resulting from anthropogenic activities
 - Chemical pollutants (e.g., fungicides, herbicides, pesticides, antifouling agents, antibiotics, synthetic hydrocarbons)
 - Synthetic radionuclides

LIST OF FIGURES

Figure 1. Proposed strategy for reef resource protection.

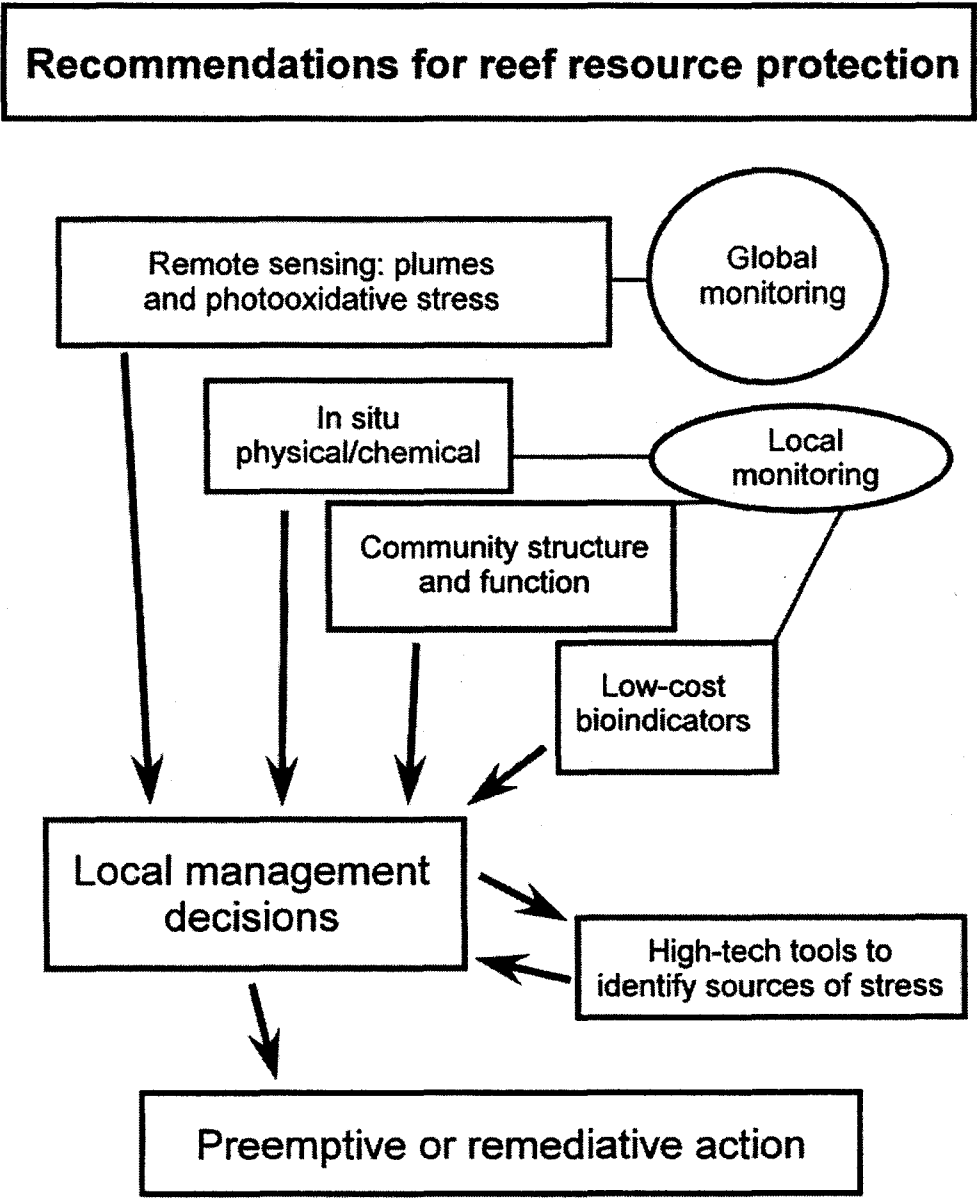


Figure 1

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Thanks to Tye Petty, Shawn Polson, Sara Polson and Samantha Ryan for preparing the Briefing Book. Your long hours and hard work are much appreciated.



Geoff Scott et al (2002)

Vitellogenin - protein - newly discovered to be in ~~forals~~ ^{faulds}
usually found in females - assoc. w/ repro.

preliminary suggestion of feminization in fish at Alina's Rf,
as compared w/ K.L. rfs

Caveat Small data set, "normal" levels unknown