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Understanding the Stress Response of Corals and *Symbiodinium* in a rapidly changing environment

Proceedings

May 10 – June 3 2005

Unidad Académica Puerto Morelos,
Instituto de Ciencias del
Mar y Limnología, UNAM
Mexico

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Chair, Targeted Research Group on
Coral Bleaching and Related Ecological Factors
(Bleaching Working Group)

Workshop 1 in a series coordinated by
Targeted Research Group on Coral Bleaching
and Related Ecological Factors

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Introduction to workshop

The inaugural workshop for the World Bank/GEF Targeted Research Group on Coral Bleaching and Related Ecological Factors (BWG) was held from May 10 - June 3 2005 in Puerto Morelos, Mexico. This workshop is the first of a series designed to galvanize the international scientific community around problems, gaps and solutions with respect to the global issue of coral bleaching and related ecological disturbances to coral reefs. The Bleaching Working Group (BWG) was originally founded by the Intergovernmental Oceanographic Commission (IOC) of UNESCO in April of 2001. The group was then incorporated as one of the six targeted working groups within the World Bank/Global Environment Facility (GEF) Coral Reef Targeted Research Program coordinated by the University of Queensland. The BWG's initial efforts included the investigation of whether there are specific indicators for coral bleaching and its effects on coral reefs. Subsequently, it expanded its mandate to examine specific physiological mechanisms for coral bleaching as well as the local ecological factors that cause bleaching and its after-effects (e.g. coral recovery), and differences between direct human stresses and those related to climate change.

The current members of the working group are:

- Ove Hoegh-Guldberg, Australia (Chair)
- Yossi Loya, Israel, (Co-Chair)
- William K. Fitt, USA
- John Bythell, UK
- Robert van Woesik, USA
- David Obura, Kenya
- Roberto Iglesias-Prieto, Mexico
- Ruth Gates, USA
- Michael Lesser, USA
- Ron Johnstone, Australia
- Tim McClanahan, Kenya
- Christian Wild, Germany

The Mexican workshop included several themes which involved invitations to leading researchers to participate in discussions and experimental components.

- May 10-13: Pulse Amplitude Modulation Fluorescence and the Stress Biology of Reef-Building Corals
  (coordinated by Roberto Iglesias-Prieto and Peter Ralph)
- May 15-17: Diversity, flexibility, stability, physiology of *Symbiodinium* and the associated ecological ramifications. (coordinated by Ove Hoegh-Guldberg and William K. Fitt)
- May 19-21: Exploration of the Coral and *Symbiodinium* genomes
  (coordinated by William Leggat, Sophie Dove, David Yellowlees)
- May 22: Coral Reef Targeted Research Working Group joint field methods
  (coordinated by Robert van Woesik)
- May 24-26: Integrated research on coral bleaching and disease
  (coordinated by John Bythell, Drew Harvell)

After reviewing progress in each of these theme areas, participants focused on key issues that arose from the research and addressing preconceived ideas in the of the reef management community. The attendance of local scholars and students at these international workshops, aided in the dissemination of the latest insights into the phenomenon of coral bleaching and related ecological impacts to the coastal community of the Mexican Yucatan coast. The research projects that were undertaken during the workshop are currently being published in the reviewed scientific literature. These proceedings include all scheduled papers. Please note

that papers by several seminars by special invitees such as Professor Michael Kuhl and Mr Tom Oliver are regrettably not captured by these proceedings.

Ove Hoegh-Guldberg

Workshop coordinator *and*
Chair, Bleaching Working Group
Coral Reef Targeted Research Project

Acknowledgements

The Bleaching Working Group is very grateful to Dr. Adolfo Gracia, Director of the Instituto de Ciencias del Mar y Limnologia at Universidad Nacional Autonoma de Mexico and Dr. Brigitta van Tussenbroek, Head of UNAM's Unidad Académica Puerto Morelos (UAPM) for hosting this workshop at their facility in Puerto Morelos. We are particularly grateful for the enormous efforts spent by Dr. Roberto Iglesias-Prieto, Dr. Susanna Enriquez, Dr. Anja Banaszak, Ms Aime Rodríguez Román, Mr. Xavier Hernández Pech and many others at the Unidad Académica Puerto Morelos (UAPM). There is clearly no way that this important workshop would have been able to prosper without these efforts. We are also grateful to the township of Puerto Morelos for their hospitality during the workshop. Lastly, we are grateful to the World Bank - Global Environment Facility Coral Reef Targeted Research Project and the University of Queensland for providing funding which enabled more than 80 participants to travel to and be accommodated during the meeting.

Participants of the workshop on "Understanding the Stress Response of Corals and *Symbiodinium* in a rapidly changing environment", May 17 2005.

Theme 1: Pulse Amplitude Modulation Fluorometry and the Stress Biology of Reef-Building Corals (May 11-13)

Pulse Amplitude Modulation (PAM) Fluorometry has become a key technique for the investigatation of changes to the photosynthetic physiology of the dinoflagellate symbionts of reef-building corals, *Symbiodinium* sp. Dr Roberto Iglesias-Prieto (Unidad Académica Puerto Morelos, Instituto de Ciencias del Mar y Limnología, UNAM) and Dr Peter Ralph (University of Technology, Sydney) coordinated a 3 day workshop focused on the use of PAM fluorometry to dectect and monitor stress in corals and *Symbiodinium*. A series of papers reviewing key aspects of the method were presented. This review of the technique was followed by hands on training sessions for researchers intending to use PAM fluorometry in their research. After the practical training session, the PAM fluorometry workshop concluded with a discussion of new technological developments in the field of PAM flourometry and the limitations of the method. The focused session on PAM fluorometry was well attended and involved over 40 participants.

Workshop participants in intense discussion following presentations. From left to right are Daniel Tchernov, Roberto Iglesias-Prieto, Tom Oliver, Mark Warner and Peter Ralph.

Sophie Dove and Susanna Enriquez discuss the finer points of light capture and photosynthesis by reef-building corals.

Introduction to PAM fluorometry

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To ensure that all attendees of the Bleaching Working Group Workshop were conversant with the techniques of chlorophyll *a* fluorescence and specifically Pulse Amplitude Modulation (PAM) fluorometry, a seminar was presented covering the basics of this technology.

Several examples of fluorescent materials, materials that absorb photons and re-emit them at a longer wavelength, were discussed. The concept of fluorescence was then considered as it applies to the PSII reaction centre when a chlorophyll molecule is excited by irradiance of either blue or red wavelengths. The amount of fluorescence will vary as a result of the condition of the operational components of the photosynthetic machinery. Following dark-adaptation, the minimum fluorescence value (Fo) of the sample can be measured. If the sample is then exposed to enough light so all the photocentres are full of photons (closed) the maximum amount of fluorescence (Fm) can be measured. The difference between these two extreme values is the variable fluorescence (Fv). Fv/Fm provides a measure of PSII photochemical efficiency.

Energy absorbed by chlorophyll *a* can either be used for photochemistry, re-emitted it at a longer wavelength as fluorescence or dissipated as heat (non-photochemical quenching). The health of the photosystems defines the relative proportions of energy directed through each of these competing pathways. The rate of electron transport is directly influenced by limitations along the electron transport chain.

Quantum yield of PSII is linked to photosynthetic activity and under some conditions is roughly proportional to oxygen production (or CO_2 uptake); however this relationship rarely holds up at elevated irradiances due to a range of competing processes including photorespiration. Electron transport is influenced by the redox state of the several critical components of PSII; primary electron acceptor (QA), the secondary electron acceptor (QB), the plastoquinone pool (PQ), PSI activity and the oxygen evolving complex.

The operational aspects of the PAM fluorometer were discussed including; light sources, lock-in amplifier, and fibre optics. Firstly, the PAM fluorometry principle is based on a 3 μs pulse of light that is synchronized to a lock-in amplifier. This allows effective quantum yield determinations to be performed in sun light, as the lock-in amplifier removes all signal not associated with the lock-in signal. The light sources available include measuring light (< 0.4 μmol photon $m^{-2}s^{-1}$), actinic light (used to drive photosynthesis, 1-2000 μmol photon $m^{-2}s^{-1}$), saturation pulse (> 6000 μmol photon $m^{-2}s^{-1}$) and far-red light (used for stimulating PSI, 730 nm). When the PAM fluorometer is being optimized for a new tissue/species it is important to set the saturating pulse width and intensity to get reproducible data. The measuring light needs to be set low enough to prevent activation of the photosystem, whilst still being able to measure sufficient fluorescence to make a measurement. A range of fibre optics are available for use with several models of PAM flurometer. , Two fibres that are relevant to coral research include the 8 mm Diving-PAM fibre useful for

assessment of tissue type or whole colonies, whereas the microfibre (50-100 mm fibre) as linked to the Microfibre-PAM is more appropriate for assessment of the photosynthetic condition of microscale habitats (polyp and coenosarc scale). Plastic fibre-optics have a higher attenuation, so you'll always have a lower signal than with a glass fibre; however the cost difference is substantial. Imaging-PAM now provides a high-resolution assessment of the spatially complex regions of corals. It is important to remember that once the optical geometry has been set and the fluorometer has been adjusted with the off-set to zero then the fluorescence signal needs to be > 130 and < 1000 units for the best quantum yield estimates. The digital gain can be adjusted to increase the fluorescence signal; however the noise also increases, so it doesn't increase precision. To ensure published data can be independently evaluated, I recommend that all data set are published with a single line of data about the PAM settings, this will allow others to attempt to replicate the experiment. For a "typical" coral, the "typical" Diving-PAM settings would be measuring light 8, saturating intensity 8, saturating width 0.6s, gain 2 and damping 2.

The biophysical condition of the PSII reaction centre was discussed in relation to maximum and minimum fluorescence. Minimum fluorescence occurs when the PSII reaction centres are fully open. Maximum fluorescence occurs when the PSII reaction centres are closed. A decrease in Fm' (light adapted maximum fluorescence) is usually linked with non-photochemistry. The following formulae can be used to assess the relative condition of the photosynthetic apparatus. Maximum quantum yield requires the coral to be dark-adapted for at least 10 min (caution that anaerobic conditions can develop), while effective quantum yield can be measured in ambient light.

Maximum quantum yield = (Fm-Fo)/Fm = Fv/Fm
Effective quantum yield = (Fm'-F)/Fm' = ΔF/Fm'

A rapid light curve is a tool for assessing the capacity of the photosynthetic tissue when exposed to series of rapidly (10 s) changing light climates. A RLC is not a photosynthesis irradiance (P-E) light curve, as the tissue does not reach steady state during each incubation. A RLC should not be interpreted as a P-E curve. RLC work best where ambient irradiance is rapidly fluctuating. RLC estimate the relative electron transport rate (rETR) at each of the irradiances. The utility of the rapid light curve was discussed amongst several of the delegates. rETR = ΔF/Fm' x PAR, where effective quantum yield is multiplied by the irradiance. This is a relative estimate of electron transport. Maximum relative electron transport rate as defined as the highest rate of ETR once the curve is fitted to an exponential decay function.

Quenching analysis was described and not discussed. This form of fluorescence analysis has utility when considering the protection and recovery aspects of photoinactivation and/or down regulation of coral.

The Photosynthetic Apparatus of *Symbiodinium.*

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Invertebrates symbiotic with dinoflagellates in the genus *Symbiodinum* are among the most important primary producers in coral reefs. In these environments they are responsible not only for the high gross production, but also for the construction and maintenance of the reef structure. Despite the importance of this symbiosis, our knowledge about the biochemical organization of the photosynthetic apparatus of dinoflagellates is still incomplete. This gap in our understanding is particularly important in the context of the study of the photobiology of thermal stress. We have commonly relied on the use of green plant models to make interpretations of chlorophyll *a* fluorescence kinetic data from *Symbiodinium*. Although in some cases these models can explain the experimental observations, caution should be taken before ascribing photosynthetic phenomenon to *Symbiodinium* that have not been characterized empirically in dinoflagellates, such as the induction of state transitions and their putative role in photo-protection.

Chromophores

The functions of photosynthetic pigments are to capture photons, transfer excitation energy to the reaction centers where primary photochemistry takes place, and, in some cases, provide photo-protection), The light harvesting function in dinoflagellates is performed by Chl c_2 and peridinin, in addition to Chlorophyll *a* (Chl a. Chlorophylls are porphyrin derivatives that form a cyclic tetrapyrrol with a chelated Mg atom ligated at the center of the macrocycle (Scheer 1991). The spectral characteristics of these molecules depend on the side groups attached to the macrocycle. In contrast with other chromophyte algae, dinoflagellates contain only Chl c_2, and their diagnostic carotenoid, peridinin. This carotenoid is capable of transfering excitation energy to Chl *a* with efficiencies close to 100%, and therefore plays a major role in light collection. All functional photosynthetic pigments are non-covalently bound to specific proteins forming Chl –protein complexes. The function of the protein moiety is to orient and space the chorophores to assure the the excitation eneregy is efficiently transfered to the reaction centers. Functionally, Chl-protein complexes are divided into light harvesting complexes or antennae and their reaction centers.

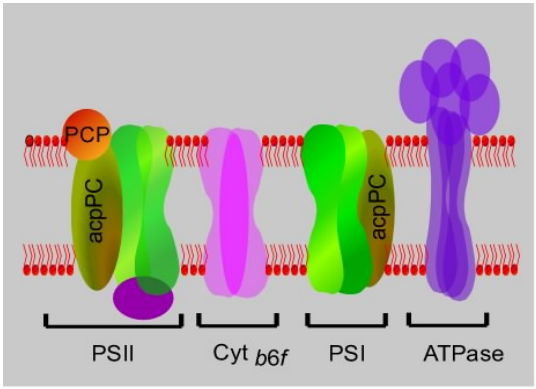

Fig 1. Schematic representation of the photosynthetic apparatus of *Symbiodinium*.

Light harvesting complexes

Dinoflagellates posses a unique light harvesting apparatus composed of the water-soluble PCP (Peridin-Chl *a*-Protein) and a transmembrane system called acpPC (Chl *a*-Chl c_2-Peridin Protein Complexes). PCP was one of the first light-harvesting complexes to be isolated and it is one of the best characterized, including a detailed structure based on X-ray crystalography (Hofmann et al. 1996). Native PCP shows apparent molecular masses between 35 to 39 kD. Analyses of the apoproteins indicate that they can occur as either monomers of about 31-35 kD or as homodimers of 14-15.5 kD. Immunological characterization of the quaternary structure of PCP taken from different species of *Symbiodinium* showed that some species contain only the monomeric or the dimeric form whereas others simultaneously presented both forms. PCP apoproteins are encoded by a family of nuclear genes. Chromophore analyses of PCP indicate the presence of variable Chl *a*: peridinin stoichiometries ranging form 2:8 to 2:12 (Iglesias-Prieto 1996).

The use of density gradient centrifugation to fractionate thylakoid membranes solubilized with glycosidic surfactants allowed the isolation of three distinct fractions comprising up to 75% of the cellular Chl *a* content, maintaining efficient energy transfer. The vast majority the accessory pigments dinoflagellates are bound to are in the intrinsic acpPC (Iglesias-Prieto et al. 1993) These complexes have Chl *a*:Chl c_2:peridin with a molar ratio of 7:4:12, and contain the majority of the xanthophylls involved in photo-protection. The content of these xanthophylls is variable depending on the prevailing light conditions (Iglesias-Prieto & Trench 1997).

Reaction centers

The other two fractions isolated include a yellow band that may to be related to photosystem II (PSII), but shows inefficient energy transfer and a photosystem I (PSI) enriched fraction. The PSI-enriched fraction contains very little amounts of Chl c_2 and peridinin. This fraction exhibits spectroscopic and kinetic properties similar to PSI isolated from green plants although there are significant differences. In *Symbiodinium* the low temperature florescence emission spectrum shows a shoulder at 709 nm instead of the characteristic peak at 730, and polyclonal antibodies raised against the PSI core of green plant fail to recognize any polypeptide in this fraction. Despite many efforts, the core of PSII in dinoflagellates has not been isolated and characterized although antibodies specific to the core polypetides from green plants cross-react with *Symbiodinium* preparations (Warner et al. 1999).

Conclusions and future directions

During the last 10 years very little progress has been made regarding the study of the structure of the photosynthetic apparatus of dinoflagellates. This information is needed if we are to describe the initial responses of these organisms to thermal and light stress. The combined use of modern genomic approaches with traditional biochemical techniques can result in significant progress in our understanding of the function and regulation of the photosynthetic apparatus *Symbiodinium* under diverse environmental scenarios in the near future.

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Multiple scattering on coral skeleton enhances light absorption by symbiotic algae

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New examination of the optical properties of intact corals

The evolutionary success of scleractinian corals as reef-builders relied on the formation of mutualistic symbioses with photosynthetic dinoflagellates. Algal photosynthesis provides nutritional advantages to scleractinians, as the translocation of photosynthates may account for their entire metabolic needs, while promoting rapid calcification. Therefore, symbiotic reef-building corals depend heavily on the efficiency with which they collect solar energy. In spite of the significant progress that coral photobiology has achieved over the last 20 years, the optical properties of intact corals have not been directly assessed yet. A major obstacle for the study of the optical properties of intact corals is the complexity of the coralline structure, consisting of coelenterate tissue, endosymbiotic algae and the intricate geometries of the aragonite coral skeleton. We provide in this work measurements of the absorption spectra of intact corals for the first time. We selected the Caribbean scleractinian *Porites branneri*, because the morphological characteristics of this species permits the preparation of even and thin coral laminae (thickness = 3 ± 0.1 mm) with homogeneous pigmentation suitable for spectroscopic analyses. During a natural bleaching event we collected specimens exhibiting a broad variation in symbiont and chlorophyll *a* content per unit of surface area (Chl *a* density), which allowed us to assess the effect of such variability on the optical properties of *P. branneri*.

Methodology employed here

Absorption spectra of the coral laminae were recorded between 380 and 750 nm with 1 nm resolution, with an Aminco DW2 (USA) spectrophotometer controlled by an OLIS (USA) data collection system. Skeleton laminae were used as reference. The light beams of the spectrophotometer were baffled with black tape apertures to match the exact dimensions of individual samples. Reflectance spectra of corals and skeletons were measured between 400 and 750 nm with 1 nm resolution using a 4800S Lifetime spectrofluorometer (SLM-Aminco, USA) equipped with a red sensitive photo-multiplier tube (R955, Hamamatsu, Japan). The use of thin laminae allowed us to obtain high quality absorption spectra of intact coral surfaces. As a result of bleaching, we had available a series of absorption spectra of specimens whose Chl *a* density varied from 3.3 mg m^{-2} to 102,1 mg m^{-2}. The observed 30-fold variation in Chl *a* density resulted in an approximately 5-fold variation in coral absorptance. Measurements of light absorption on transmission mode are not only laborious, but can be difficult to implement with corals of other morphologies. We propose as an alternative technique for estimating coral absorption, the determination of reflectance spectra, since the inferred absorption spectrum compared well with those obtained in transmission mode.

Variation in light absorption properties as a function of coral pigment content

Determination of absorptance as a function of the variation in pigment content showed that the light-harvesting capacity of *P. branneri* decreases abruptly only for Chl *a* density below 20 mg m^{-2}, remaining practically constant for values above this threshold. These results differ from former estimations based on filtered coral slurries[1-4].

To quantify the variations in pigment light-absorption efficiency, we estimated the changes in the chlorophyll *a* specific absorption coefficient (a*) as a function of Chl *a* density. The analysis of this variation indicates that the values of a* estimated for intact corals are between 2 and 5 times higher than those estimated from suspensions of freshly isolated symbionts with similar pigment density. On the other hand, the a* values obtained in our study for a suspension of freshly isolated symbionts are consistent with measurements of the absorption of filtered blastates[1-4], and with values reported for phytoplankton[5]. The increase in the absorption efficiency may be understood through simple physical considerations. In simplified form, a coral structure may be visualized as a thin layer of small, pigmented particles, above one dimensional surface of coral skeleton. As the illumination reaches the pigment layer a fraction of the incident light is absorbed ($\Phi_{abs}^{(i)}$). Part of the light transmitted through the layer is backscattered by the skeleton and passes again ($\Phi_{abs}^{(s)}$) as diffuse light through the layer of pigment, increasing thus the capacity of light absorption by the particles. This theoretical model concludes that a flat scattering surface can enhance the absorption of the particle by a factor of up to 3 (for a non absorbing surface, $\Phi_{abs} = \Phi_{abs}^{(i)} + \Phi_{abs}^{(s)} = (1+2R)\Phi_{abs}^{(i)}$). The model becomes theoretically intractable when the particle is placed inside a concavity or is exposed to several reflective coral skeleton surfaces. Nevertheless, it concludes that light absorption by the particle could be amplified by a factor much higher that 3.

The comparison done in the North Queensland Tropical Museum (Townsville, Australia) on 56 coral skeletons of *Favide* spp, and 18 spp of other massive taxonomic families, reveled a large variability among species in the variation of the maximum enhancement factor. We found values from a minimum of 2.9 showed by *Caulastrea curvata* to a maximum of 8.3 showed by *Cyphastrea japonica*. It is noteworthy that the three *Porites* spp from the Indo-Pacific examined showed similar values than the maximum value estimated in this work for *Porites branneri*. We concluded that coral skeletons are efficient bulk scatterers allowing to enhancing the capacity of light absorption by algal pigments through the diffusive propagation of light over longer optical paths. Multiple scattering by the coral skeleton provides diffuse and homogeneous light fields for the symbionts reducing pigment self-shading.

Biological and ecological implications

The biological and ecological implications of the optical properties of the intact coral structure revealed by this study are diverse. We concluded that: a) the light fields within coral tissue are not predictable from the water column light attenuation descriptions, and are very dependent on pigment density; b) the study of photoacclimatization of different species of corals and the propagation of the thermal stress needs to be revised from this new perspective; c) the modulation of the internal light field by the coral skeleton may be an important driving force in the evolution of symbiotic scleractinian corals; and d) determinations of the minimum quantum requirements for symbiotic scleractinian corals need to be re-assesed. Our results indicate that symbiotic corals are one of the most efficient solar energy collectors in nature. These organisms are capable of harvesting the same amount of incident radiation as the leaf of a terrestrial plant with six times less pigment density.

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An overview of the interpretation and current use of chlorophyll fluorescence to understand coral bleaching

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As the thermal sensitivity of some zooxanthellae is seen as one of the primary causes of coral bleaching, many laboratories have undertaken intensive studies in the photobiology of these symbiotic dinoflagellates to better understand how photosynthetic processes may be affected by excessive thermal and light exposure. Several methods of measuring active chlorophyll fluorescence to infer photosynthetic function are becoming widely used in coral biology and for assessing photosystem stress during experimental or natural bleaching in particular. The saturation pulse method commonly used with the pulse amplitude modulation (PAM) fluorometer was introduced to the field of coral biology almost a decade ago, and has become a common tool for many reef biologists investigating coral bleaching. While the method and the data that it can quickly generate has proven quite beneficial in extending our fundamental understanding of the photobiology of zooxanthellae symbioses, it is not without certain caveats that should be addressed in light of current efforts to better understand the biochemical and cellular pathways involved in coral bleaching.

Importance of experimental design and key parameters

While core proteins and pigments within the reaction centers of photosystem I and II (PSI & PSII hereafter) are highly conserved across all known photosynthetic eukaryotes, there are many differences at the level of light harvesting complex structure and function which can and do affect commonly used fluorescence parameters. It is prudent to note that the much of the theoretical groundwork that forms the basis of using PAM fluorometry extends from work with higher plants which can represent a significant departure from the physiological variability one is likely to encounter in working with algal groups (including the dinoflagellates) that extend from the red algal lineage. A second important point is that there are many differences in current designs of coral bleaching experiments utilizing PAM fluorometry such that results should be evaluated in light of the scale of the design itself. It is important to view current results in relation to the ecological relevance of the experimental design versus the degree of physiological reduction. The field of chlorophyll fluorescence contains a semantic minefield of terminology that can be an unnecessary source of confusion when comparing different bleaching experiments, and one should strive to follow previously published guidelines for correct use of such terms (see Kromkamp and Forster 2003). Some of the more common parameters in use today are the dark acclimated quantum yield of PSII (Fv/Fm), the effective quantum yield of PSII (ΔF/Fm'), photochemical (qP) and nonphotochemical fluorescence quenching (qN and NPQ), and electron transport rate (ETR).

Fv/Fm and ΔF/Fm' are two of the most common parameters used for rapidly assessing the status of PSII in zooxanthellae within corals, as they are easy to measure and have a long history of use in plant and phytoplankton biology for detecting photoinhibition. Corals exposed to thermal stress as well as those sampled during natural bleaching events have shown a significant loss in Fv/Fm compared to corals held in non-bleaching conditions which typically precedes detection of any loss

in zooxanthellae density. Declines in Fv/Fm are correlated with the loss of PSII D1 protein content in some cases (Warner et al. 1999; Lesser and Farrell 2004), thereby enforcing the idea of thermal stress exacerbating a pathway of cellular damage seen in light enhanced photoinhibition studies. However, more work is needed to fully establish if this correlation holds across different species of zooxanthellae. For example, some zooxanthellae could possibly show a loss in PSII activity while degradation of reaction center proteins is impeded. Likewise, an important point is that one should be careful to delineate stress related loss of Fv/Fm with possible seasonal declines in Fv/Fm that largely reflect photoacclimation as opposed to chronic cellular stress. Similarly, one can use the effective quantum yield (ΔF/Fm') or PSII capacity in the light acclimated state as a proxy for stress, so long as one has an understanding of the typical patterns of diurnal decline and recovery due simply to increased levels of light energy dissipation during daylight hours (i.e. enhanced quenching of the fluorescence signal due to non-photochemical pathways such as xanthophyll cycling) in their organism of interest. Recent work has shown that analysis of ΔF/Fm' can also indicate when a bleaching experimental design may be inducing more artificial chronic stress than would be seen in the field (see Franklin et al. 2004, Fig.1, for a good example). A second point of caution is that ΔF/Fm' is dependent on the previous light history of the alga and one should take care in interpreting results for experiments that involve acute light shocks that may not replicate physical conditions typically seen in the field. In this same vein, a fluorescence induction curve conducted with a light intensity significantly higher than that used in the experimental treatment can yield large decreases in ΔF/Fm' which reflect an artefact of the light intensity used for the induction curve rather than how a coral may have processed light under the experimental conditions. Of the quenching parameters typically measured, photochemical quenching (qP) is typically the hardest to measure under field conditions. This difficulty is due to the fact that the equations traditionally used to calculate this variable rely on accurate measurement of any quenching of the initial fluorescence signal (F_o), which can happen quite frequently in zooxanthellae (personal observation). Proper assessment of qP requires the ability to rapidly darken a sample and apply a pulse of far-red light to re-oxidize PSII traps. Such protocols are not available on current submersible instrumentation (e.g. the diving PAM), and alternative equations developed with higher plants to circumvent the need to know F_o for qP calculation have proven to be less accurate for corals (personal observation).

Other parameters, such as excitation pressure over PSII (*sensu* Iglesias-Prieto et al. 2004) and NPQ have shown to provide a good proxy for potential thermal stress in some corals. The central idea to this point is that some thermally sensitive zooxanthellae may have less capacity to dissipate excess energy than others, and their homeostatic level of excitation pressure is higher than that of other zooxanthellae that show greater thermal resistance. On the other hand, more work is currently underway to better understand how (or if) any compensatory electron turnover at PSII may also play a role in explaining how some corals with elevated NPQ or excitation pressure can maintain PSII function during thermal stress.

Lastly, electron transport rate or ETR has been used heavily to infer changes in photosynthetic activity in general and during thermal and/or light stress. While plotting ETR vs. irradiance is a common practice one should take great caution in interpreting such data using traditional photosynthesis to irradiance terminology and concepts, as they are not synonymous in many cases. For example, one should not assume that maximal ETR is representative of maximal photosynthetic rate (P_{max}) as measured by other methods (e.g. respirometry). Comparisons of gross photosynthesis and ETR in several groups of algae have shown that these two variables co-vary only within a range of light intensities and that they can significantly

depart from each other at higher levels of light (Geel et al. 1997). Such an effect is most likely due to non-assimilatory electron flow through PSII such as that due to Mehler activity, which can change between different algae or under different physical conditions. Likewise, current evidence suggests that coral absorptance can change substantially during bleaching (Enriquez et al. 2005), thus rendering any measurement of "relative" ETR that does not account for such absorptance changes highly suspect to gross error.

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Diel Cycling of Nitrogen Fixation in Corals with Symbiotic Cyanobacteria

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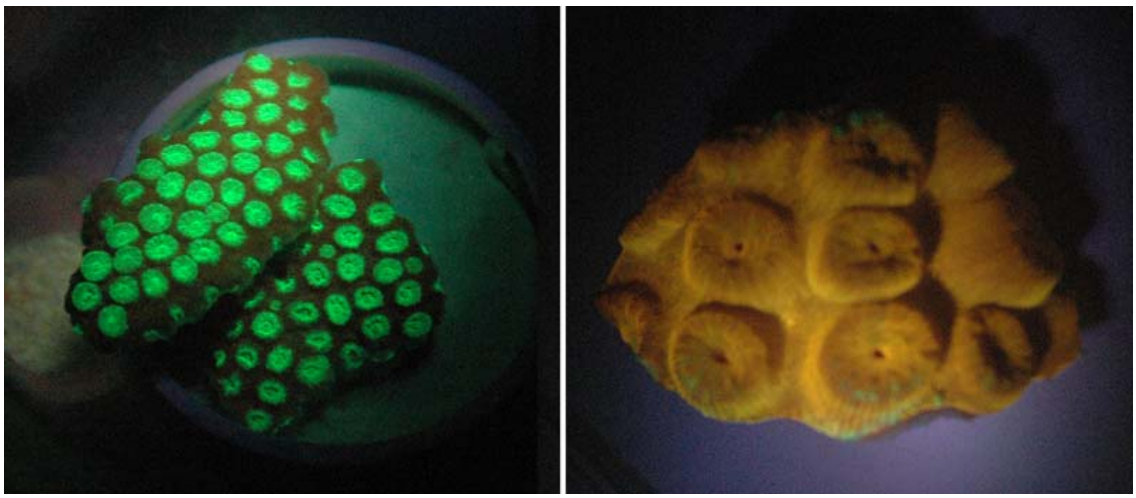
Corals in mutualistic symbiosis with endosymbiotic dinoflagellates (zooxanthellae) are essential components of the ecological diversity of tropical coral reefs. Zooxanthellate corals also exist in an environment where inorganic nitrogen limits the growth and abundance of zooxanthellae *in hospite*[1-3]. Many colonies of the Caribbean coral, *Montastraea cavernosa*, contain endosymbiotic cyanobacteria[4]. These cyanobacteria co-exist with the zooxanthellae and express the nitrogen fixing enzyme nitrogenase[4]. Here we show that the percentage of colonies containing symbiotic cyanobacteria increases with increasing depth, and that measurements of nitrogen fixation show a diel pattern with the highest rates of nitrogen fixation in the early morning and evening. No nitrogen fixation was measurable in non-symbiotic con-specifics. The δ^{15}N stable isotope data show a strong nitrogen fixation signal in the zooxanthellae fraction of corals with cyanobacterial symbionts suggesting that zooxanthellae use fixed nitrogen products. The timing of nitrogen fixation avoids maximum periods of photosynthesis to avoid severe hyperoxia, and nitrogen fixation does not occur when the coral experiences hypoxia or anoxia. These cyanobacteria require low oxygen tensions to support the respiratory processes that provide the energy required to fix nitrogen. Nitrogen fixation in these corals provides an important supplemental source of a limiting element for this novel microbial consortium.

Figure 1. Green-brown (left) and red morphs of *Montastraea cavernosa* under Blue light and viewed through an orange filter set. Photo: O. Hoegh-Guldberg



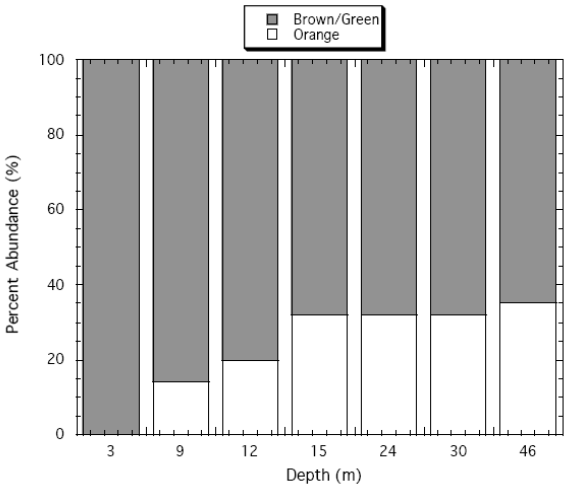


Figure 2 Percent abundance of Montastraea cavernosa colonies containing endosymbiotic cyanobacteria at different depths around Lee Stocking Island, Bahamas. Significant differences were observed with depth using a contingency table analysis; Likelihood ratio, $\chi 2$ =18.17 P=0.006, Pearson, $\chi 2$ =14.065 P=0.03. Post-hoc multiple comparison testing showed that the population of orange M. cavernosa were significantly greater deeper (≥15 m) compared to shallow depths (≤ 12 m).

The presence of cyanobacterial symbionts that can fix nitrogen in zooxanthellate corals represents not just a novel microbial consortium of photosynthetic eukaryotes and prokaryotes, but one that challenges the long-term paradigm that nitrogen is limiting in corals. Our observations indicate that several species of scleractinian corals in the Caribbean and on the Great Barrier Reef have individuals harboring stable, non-pathogenic populations of endosymbiotic cyanobacteria. If the occurrence of this consortium in other species of scleractinian corals residing in oligotrophic tropical waters is more common than previously believed, it could provide an important supplemental source of a limiting element for zooxanthellate corals. These inputs of new nitrogen from endosymbiotic cyanobacteria in corals not only has important implications for our current understanding of the role of nitrogen as a limiting and regulatory element in these associations, but also requires that we re-examine the role corals play in the nitrogen budgets of coral reefs. Because corals release large quantities of dissolved organic material containing high concentrations of both organic and inorganic sources of nitrogen the implication of corals as nitrogen fixes consortiums for the biogeochemical fluxes of nitrogen in carbonate sands and pore waters of coral reefs are potentially very large.

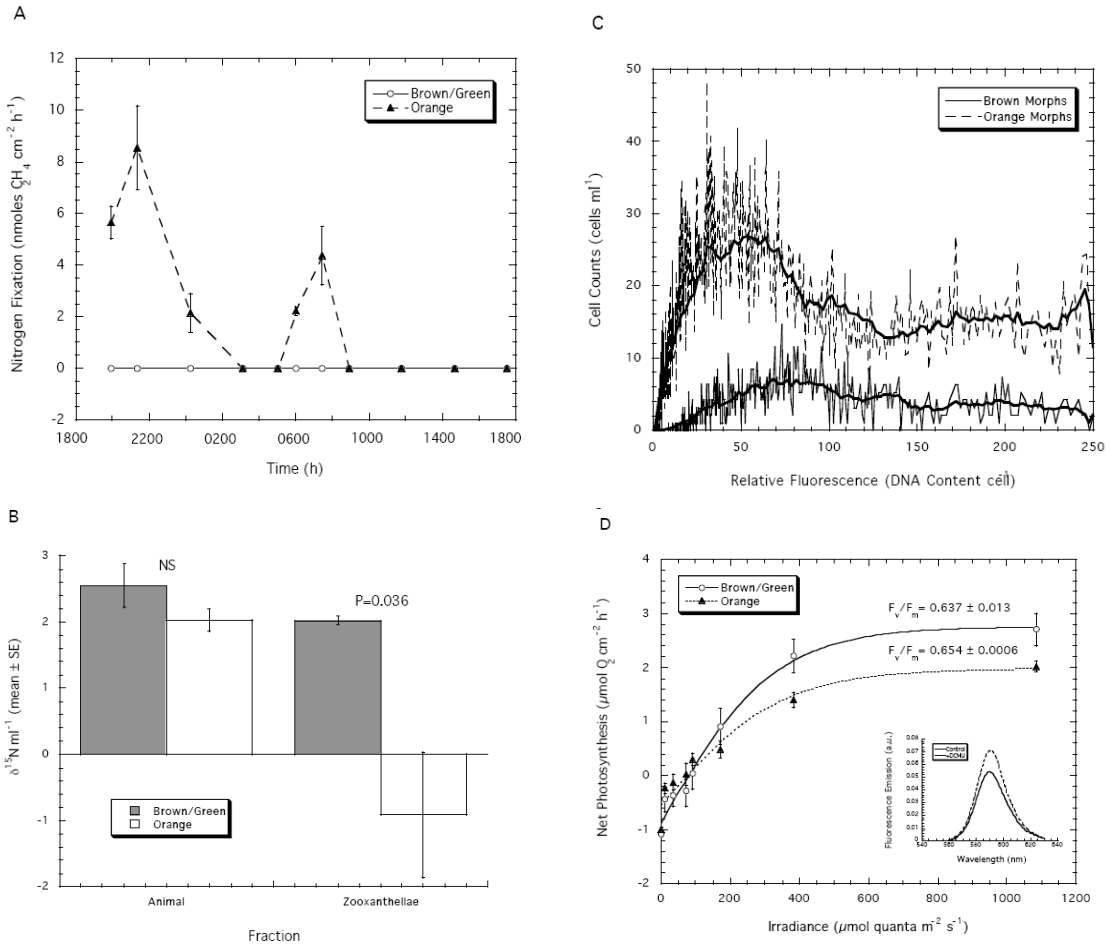


Figure 3. a) Nitrogen fixation (acetylene reduction) brown/green (N=3) versus orange (N=3) colonies of M. cavernosa. There were significant effects of colony type (ANOVA: P<0.0001), time (ANOVA: P<0.0001), and the interaction of colony type and time (ANOVA: P<0.0001), Post-hoc multiple comparisons show that there were significant effects of time (SNK: P<0.05) between colony types. b) Stable δ15N isotope results for brown/green (N=3) versus orange (N=3) colonies of M. cavernosa. There were no significant effects of colony for the animal fraction but a significant effect (ANOVA: P=0.036) of colony was observed for the zooxanthellae fraction. c) Cellular DNA content measured as relative fluorescence of Picogreen staining on isolated zooxanthellae from brown/green (N=3) and ornage (N=3) colonies of M. cavernosa. Solid line through distribution is smoothed curve for easier visualization. d) Net photosynthesis-irradiance (oxygen flux) curves for brown/green (N=3) versus orange (N=3) colonies of M. cavernosa. There were significant differences in maximum productivity (ANOVA: P<0.05, brown/green; 3.63 ± 0.22 [SE] μmol O2 cm-2 h-1, orange; 2.53 ± 0.29 [SE]), and in calculated rates of respiration (ANOVA: P<0.05, brown/green; -0.871 ± 0.125 [SE] μmol O2 cm-2 h-1, orange; -0.543 ± 0.157 [SE]), but not the 13 light-limited portion of the fitted curves. The insert is the phycoerythrin emission from the fluorescence induction on orange colonies with and without exposure to the herbicide DCMU. An approximately 22% increase in phycoerythrin emission was observed when exposed to DCMU.

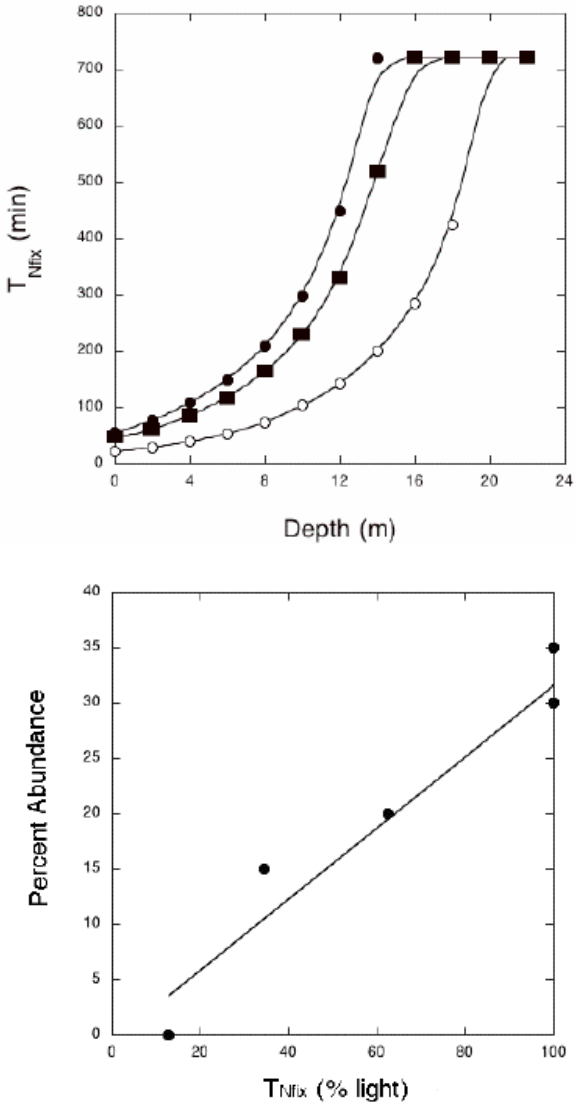


Figure 4. a) The relationship between time (min) and depth (m) during which nitrogen fixation can take place in *Montastraea cavernosa* with endosymbiotic cyanobacteria. The open circles represent predictions using the data from the fitted P-I curve. The closed squares are the simulation results using the empirical respiration rates, and the closed circles represent the simulation results under conditions where we allowed nitrogen fixation to take place at the same irradiances experienced in situ by our coral samples. b) Using the percent abundance data of Montastraea cavernosa with endosymbiotic cyanobacteria and the simulation data of the percentage of daylight irradiances that are below the compensation point (± 10%) there is a significant functional relationship (ANOVA: $P<0.05$, $y=0.32139\ (x) - 0.54562$) that predicts increasing abundances of these corals with increasing depth.

Host pigments, photosynthetic efficiency and thermal stress

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Scleractinian corals provide the calcium carbonate matrix of coral reefs due to efficient photosynthesis by endosymbiotic dinoflagellates. The ability of these dinoflagellates to harvest solar energy from within the host tissue is essential for, yet dangerous to, the success of scleractinian corals as over-energization of the photosynthetic units results in the formation of reactive oxygen species (ROS), which cause cellular damage. We have investigated the hypothesis that host pigments may act as an extension of the dinoflagellate photosynthetic pigments to absorb light for utilization by phtosythesis that Dinoflagellate or pigmentation responds over a period of days to months to changes in photon flux density (PFD) by reciprocally altering chlorophyll (Chl) and carotenoid pools (Iglesias-Prieto & Trench, 1997). In high PFD environments the carotenoid pool, especially the xanthophyll pool, is increased enabling the quenching of harvested energy to heat and the direct quenching of ROS. There are conflicting reports in the literature over whether host pigments, similar to carotenoids, act photoprotectively. Dove (2004) argues for the photoproctective nature of purple-blue non-fluorescent pigments. While Mazel et al. (2003) argue the opposite for fluorescent green "GFP-homologs" that showed no depth stratification.

Montipora monastriata from Wistari Reef (GBR, Australia) at a depth of 3-5 m occur predominantly as purple-blue, tan or brown morph in the open; and green, brown or red morph under the overhangs of the spur and grove formation. Tan morphs contain host pigments, pocilloporin and a putative green GFP-homolog; blue morphs contain pocilloporin, and green morphs contain the putative green GFP-homolog. We investigated whether the colour morphs were a response to PFD; whether photosynthetic flux measured as oxygen flux differed for the different morphs and how these rates related to the light absorption capacity of the endosymbionts within the specific host environments.

Effect of changing photon flux on host pigments

Similar to the changes in the carotenoid pool in high PFD, host pigments (pocilloporin and the putative green GFP homolog) respond to 2 months of increased PFD by increasing expression levels relative to total protein. Green cave dwelling morphs increased pocilloporin, but not "green" GFP levels that were already elevated under low PFD (Fig. 1). These results support a photoprotective role for these pigments, whilst allow for a lack of depth stratification observed for green-fluorescent GFPs.

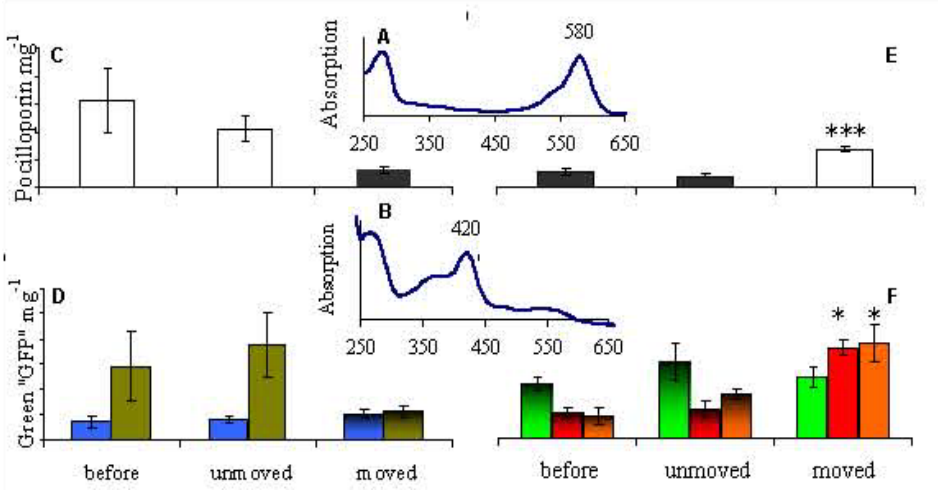

Figure 1. Effect of transplanting *Montipora monasteriata* between high (unshaded) and low (shaded) light fields on host pigments. Absorption spectrum of (**A)** pocilloporin (**B**) putative green GFP. Transplantation of blue and tan morph from high to low PFD **(C,D)**; of green, red and brown morphs from low to high PFD **(E,F).** *, $p<0.05$.

Effect of host pigmentation (Chl concentration and skeletal structure) on oxygen flux and absorption capacity by endosymbiotic algal pigments

The rate of maximum photosynthesis (P_{max}) is typically lower for low PFD (LL) versus high PFD (HL) plant and algae (Nigoyi 1999). In line with this observation, cave-dwelling *M. monasteriata* had lower P_{max} than open dwelling blue morphs (Fig. 2AB). Paradoxically, tan-HL morphs however had P_{max} that were not significantly different from the cave dwelling morphs, and at least half the value of blue-HL morphs. Oxygen flux measurements were made in January of 2002 at the onset of a major GBR bleaching (max. temp attained = 30 ^{o}C). Recently, Enriquez et al. (2005) have shown that 675 nm light absorption by algae in symbiosis can be estimated from reflectance measurement of coral surfaces. The specific absorption coefficient *a** of Chl *a* increases exponentially with drastic reductions in Chl *a*. We calculated a* for Chl *a* concentrations resulting from control and 32^{o}C heating for 6 h for blue.-HL-pocilloporin containing – morphs and brown-HL and brown-LL - lacking host pigmentation – morphs. The studied showed that this exponential rise occurred faster at higher Chl *a* densities for morphs expressing pocilloporin (Fig. 2D). Light enhancement within host tissue (as pigmentation nears zero) is due to multiple scattering by diffuse skeletal surfaces (Enríquez et al. 2005). Potentially, certain skeletal morphs are more predisposed to turn purple or blue (express host pigments) in response to a loss in algal pigmentation than others. Host pigments may function photo-protectively and facilitate algal photosynthesis under elevated internal PFD by preventing overload of the photsystem reaction centres. The mechanisms by which pocilloporin (and other pigmented GFPs) achieve this feat are yet to be determined, yet are likely to include “optical dampening” by host pigments of UVR and/or PAR. There is no paradox over P_{max} in Tan and blue open-dwelling morphs: the difference between P_{max} blue-HL and tan-HL can be assigned to the difference in Chl *a* density and hence the light fields directly experienced by algae in symbiosis (Fig. 2 B-D).

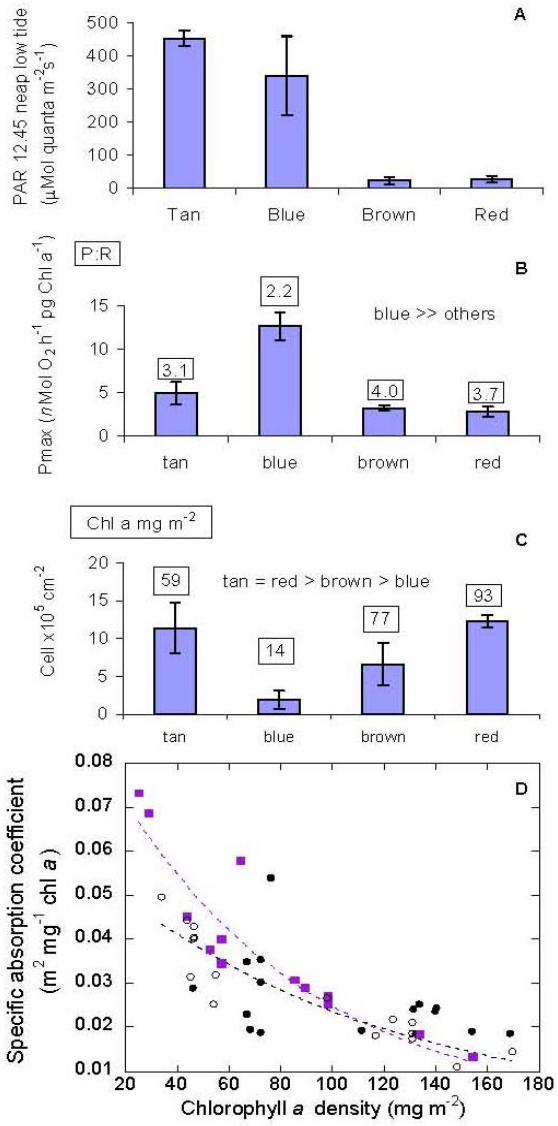

Figure 2. Typical mid-day light fields (**A**); maximum rate of photosynthesis; boxed text shows ratio of photosynthesis to respiration (**B**); areal algal cell densities; boxed text shows corresponding Chl *a* density (**C**); estimate of algal light absorption (*a**) relative to Chl *a* density for different colour morphs of *Montipora monasteriata*. ■, blue-HL morphs a* = 0.09 ± 0.01 x $e^{-0.013\pm0.002 \times [Chl\ a]}$, r^2=0.83, *p*< 0.01; ❍, brown-HL morph a* = 0.06 ± 0.005 x $e^{-0.0092\pm0.001 \times [Chl\ a]}$, r^2=0.85, *p*< 0.01, ●, brown-LL morph, no relationship.

Conclusions and future directions

Dove et al. (*in press*) showed that heating *M. monasteriata* to 32°C for 6 h, not only decreased the Chl concentration, but also the relative xanthophyll and pocilloporin pools. Dove (2004) showed that pocilloporin-rich morphs of *Acropora aspera* were photosynthetically more able to handle increases in PFD than pocilloporin-poor morphs, but that after prolonged exposure to 32-33°C, this result was reversed and coincided with elevated mortality. Corals obviously exist were water temperatures frequently exceed 33°C in the summer: are these corals capable of expressing pocilloporin and/or do they take on skeletal morphologies that minimise internal light enhancement?

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Imaging-PAM: Operation and Possibilities

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High resolution imaging of variable chlorophyll *a* fluorescence emissions was used to identify 2-dimensional heterogeneity of photosynthetic activity across the surface of corals. In comparison to earlier studies of fluorescence analysis (Ralph *et al*. 2002), the Imaging-PAM enables greater accuracy by allowing different tissues to be better defined and by providing many more data points within a given time (Hill *et al*. 2004; Ralph *et al*. 2005). The resolution of the instrument provides detail down to 100 µm and the area imaged can be controlled by the user. The standard Imaging-PAM measures an area of 3.5 x 4.5 cm and the Maxi-Imaging-PAM measures 10 x 13 cm. A micro-head attachment is also available for more detailed, fine scale investigations. An added component to the apparatus is a 96 well plate (imaged under the Maxi-Imaging-PAM) which has applications for ecotoxicological studies. This instrument contains a ring of blue, red and near-infra-red (NIR) LED's and a CCD camera for fluorescence detection. A new feature provided by this instrument enables the measurement of absorptivity = 1-(Red/NIR).

Photosynthetic responses of coral tissue to light

Images of fluorescence emission indicated that the photosynthetic activity of coenosarc and polyp tissues responded differently to changing light and diel fluctuations in *Acropora nobilis*, *Goniastrea australiensis*, and *Pavona decussata*. Fig. 1 shows variable chlorophyll *a* fluorescence images of *A. nobilis* under 295 µmol photons m^{-2} s^{-1}.

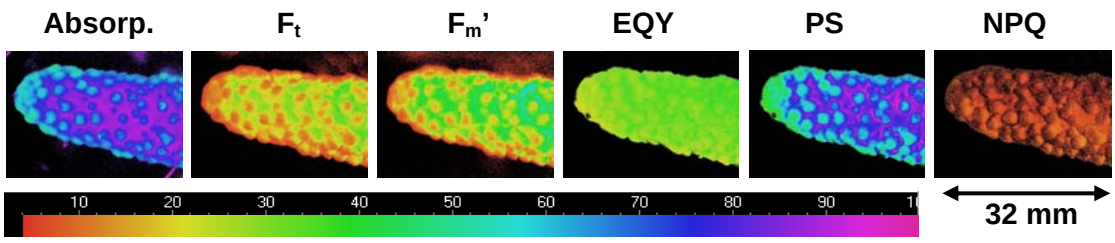

Fig. 1: Chl *a* fluorescence images of *A. nobilis* showing PAR absorptivity, F_t, F_m', effective quantum yield (EQY), relative photosynthesis rate (PS) and non-photochemical quenching (NPQ). Colour scale shown.

Diel fluctuations in F_v/F_m revealed that different tissue types showed varying degrees of downregulation/photoinhibition spatially and temporally. Upon exposure to experimentally controlled high light conditions (1000 µmol photons m^{-2} s^{-1}), downregulation of photosynthesis occurred as well as higher NPQ within the polyps of *G. australiensis* and on the polyp walls and coenosarc of *A. nobilis*.

Studying coral bleaching with the Imaging-PAM

In *Pocillopora damicornis*, *A. nobilis* and *Cyphastrea serailia* the Imaging-PAM was used to map the impact of bleaching stress. The effect of bleaching conditions (33°C

and 280 µmol photons m^{-2} s^{-1}) was studied over a period of 8 h. Marked changes in fluorescence parameters were observed for all three species. Although a decline in EQY was observed, *P. damicornis* showed no visual signs of bleaching on the Imaging-PAM after this time. In *A. nobilis* and *C. serailia*, visual signs of bleaching over the 8 h period were accompanied by marked changes in F_t (light-adapted fluorescence), NPQ and EQY. These changes were most noticeable over the first 5 h. The most sensitive species was *A. nobilis*, which after 8 h at 33°C had reached an EQY value of almost zero across its whole surface (Fig. 2). Differential bleaching responses between polyps and coenosarc tissue were found in *P. damicornis*, but not in *A. nobilis* and *C. serailia*. Spatial variability of photosynthetic performance from the tip to the distal parts was revealed in one species of branching coral, *A. nobilis*.

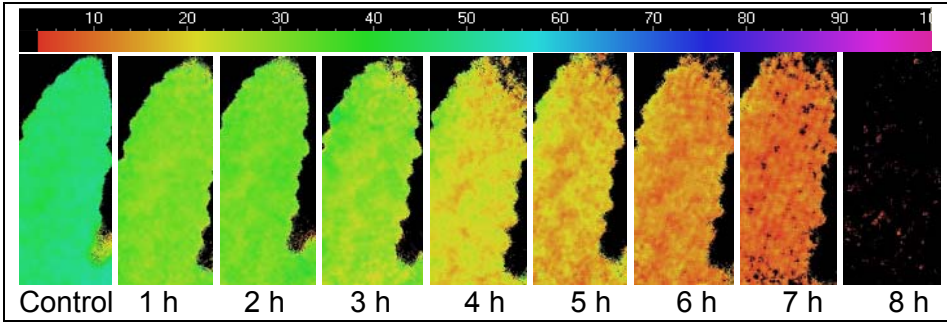

Fig. 2: EQY of *A. nobilis* over 8 h of exposure to bleaching conditions. Colour scale shown.

Photosynthetic performance of zooxanthellae within animal tissue affected by Porites Ulcerative White Spot (PUWS) Syndrome

The Imaging-PAM was also used to map the photosynthetic gradient across syndrome lesions. In this case, *Porites Ulcerative White Spot (PUWS) Syndrome* was imaged (Fig. 3). Several variable chlorophyll *a* fluorescence parameters (F_o, F_m and EQY at high irradiances (596 µmol photons m^{-2} s^{-1}) showed distinct gradients across the syndrome lesion. This suggests that in the case of PUWS, photosynthetic performance of zooxanthellae is affected and that fluorometry may be a useful tool to assess the health of the symbionts associated with coral syndromes in general.

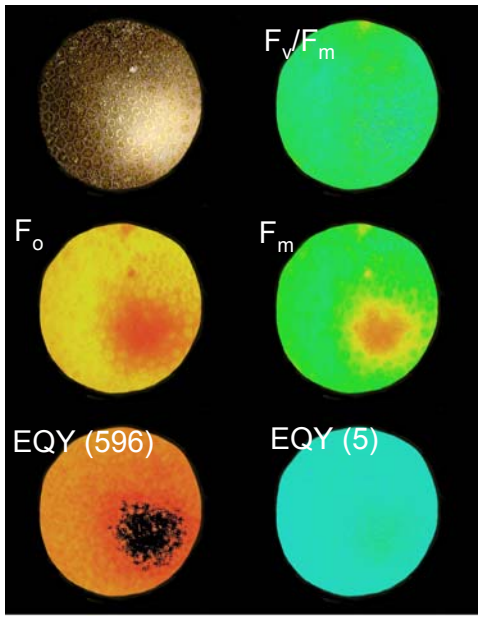

Fig. 3: Photograph and variable chlorophyll *a* fluorescence of *Porites sp.* showing F_v/F_m, F_o, F_m, EQY at 596 and 5 µmol photons m^{-2} s^{-1}.

Conclusions and future directions

The Imaging-PAM allows for a range of photosynthetic parameters to be measured across a 2-dimentional photosynthetic surface and also provides the means to measure absorptivity. The results of these experiments indicate that stress-induced photosynthetic responses are rarely continuous across a coral surface and that variations exist between the various tissue types. As a result, it is emphasised that it is unwise to extrapolate single-point measurements to a whole colony.

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Seasonal fluctuations in the physiology of *Stylophora pistillata*

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Seasonal fluctuations in the maximal quantum yield of photosynthetic electron flow through photosystem II (F_v/F_m) as measured *in situ* were found to occur naturally in zooxanthellae of non-bleaching colonies of the branching coral *Stylophora pistillata* growing at 5, 10 and 20m. These fluctuations correlated stronger with changes in irradiance than changes in seawater temperature. Seasonal fluctuations were also found in the chlorophyll *a* density, which was due mostly to seasonal changes in zooxanthellae density. Results show that during the summer of a "non-bleaching year", corals will loose 80% of their zooxanthellae (in comparison with zooxanthellae densities measured during the winter) the equivalent of zooxanthellae loss measured in the Caribbean during the 1998 mass bleaching event (Warner et al. 2002). Underwater photographs taken monthly reveal the dramatic colour changes corals go through even during "non-bleaching" years. These results shed some light on the issue of what is "real" bleaching as apposed to seasonal changes. Possibly, what is termed mass bleaching should be seen as taking this normal (seasonal) paling of coral colour one notch forwards. For future PAM fluorometry based studies, it is suggested that, in order to correlate F_v/F_m values with anthropologically caused stresses, (a) F_v/F_m measurements be performed *in situ* under natural conditions and (b) natural seasonal fluctuations in F_v/F_m be taken into account when using this parameter for diagnosing coral bleaching. It is further suggested that high irradiances may cause decreased F_v/F_m values at least as much as, if not more than, high temperatures.

Figures

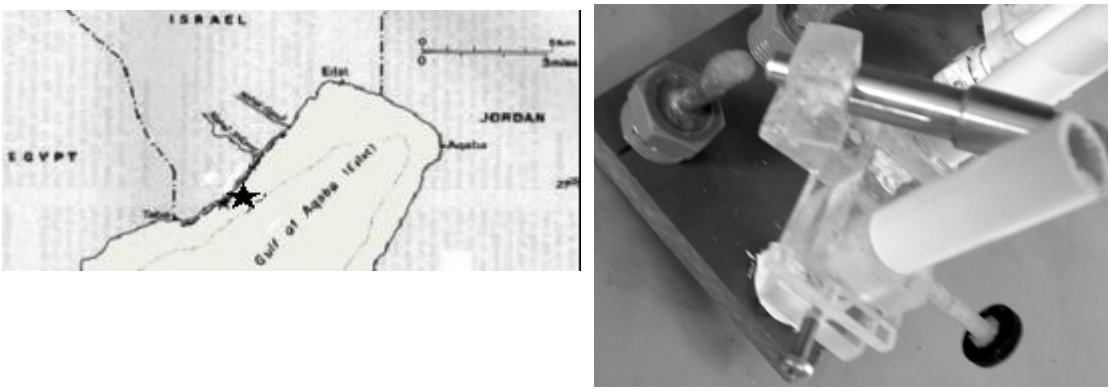

Fig.1. a) Study site and b) experimental set up: Specially made plastic holder for the Diving-PAM's probe, allowing for repetitive measurements to be performed keeping the same angle (69o) and distance (1 cm) between the sample and the PAM's probe

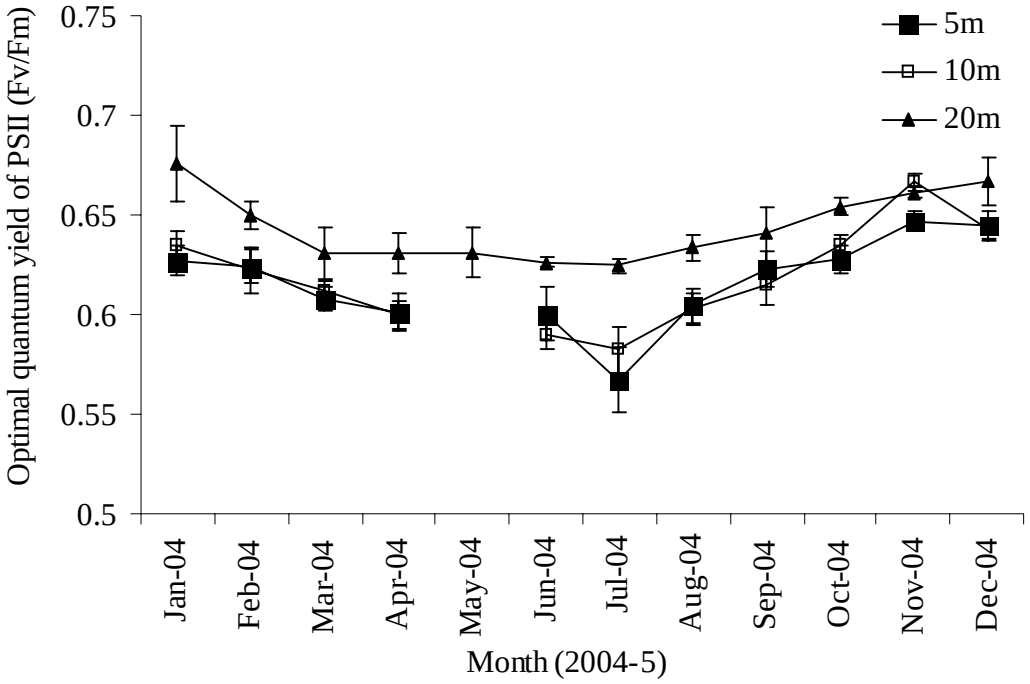

Fig. 2. Seasonal variations in F_v/F_m in *Stylophora pistillata* (n=5, ±SE) growing at 5, 10 and 20m

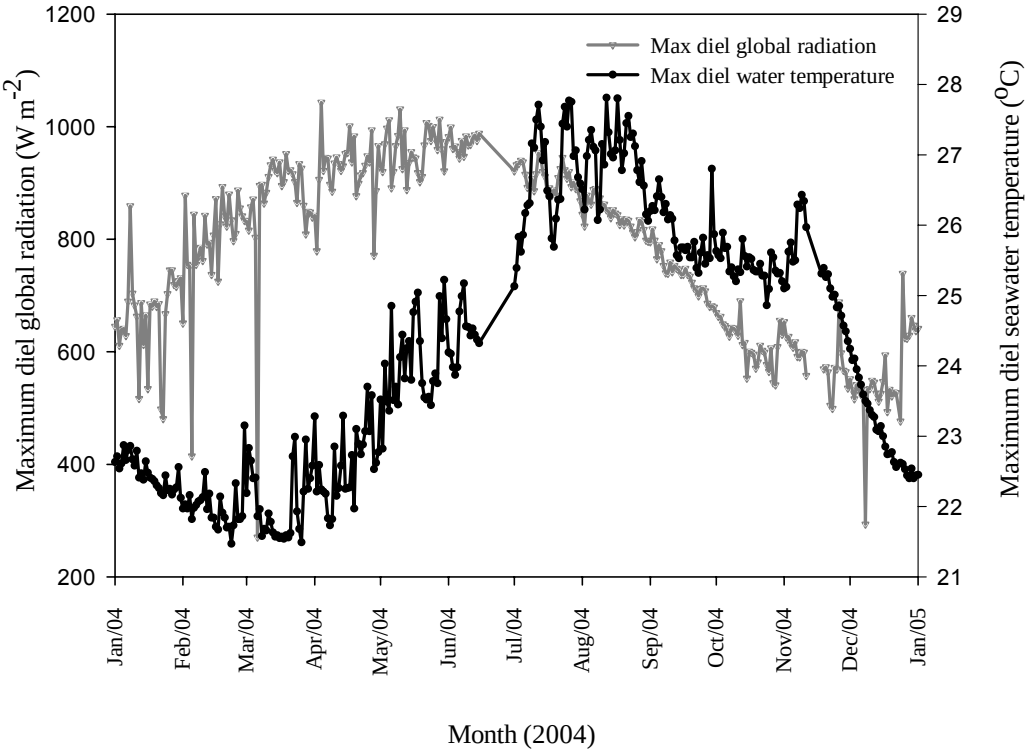

Fig. 3. Seasonal variations in global diel maximum global radiation (left y-axis) and diel maximum seawater temperature (right y-axis) for the year 2004.

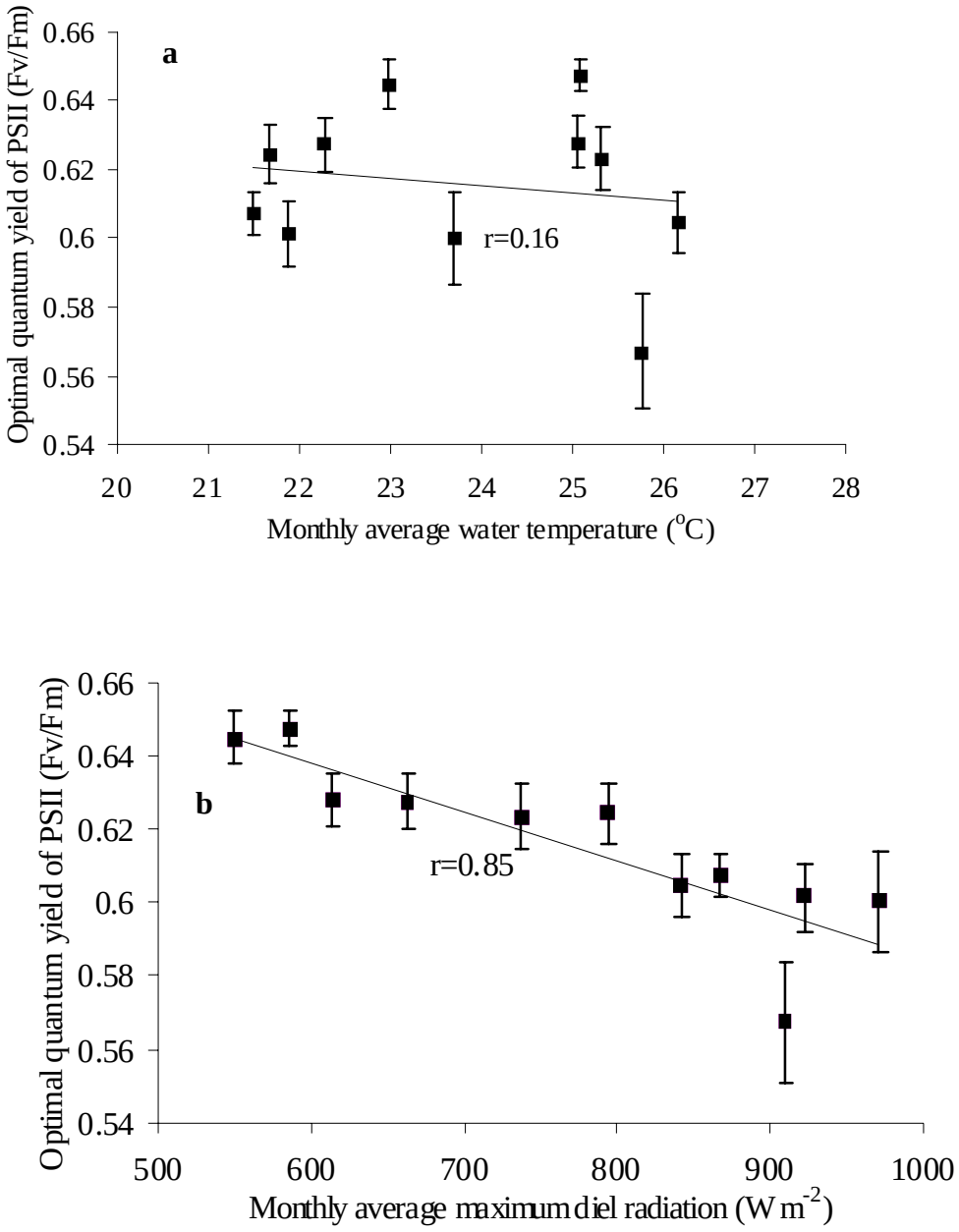

Fig. 4 Correlations between monthly F_v/F_m measurements of *Stylophora pistillata* (n=5) growing at 5m and monthly average of a) seawater temperature (°C) and b) diel maximum global radiation (W m^{-2})

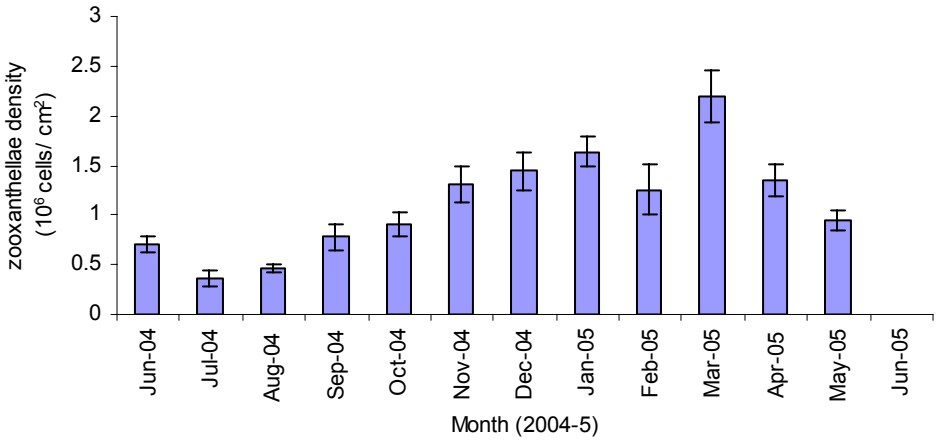

Fig. 5. Seasonal dynamics in zooxanthellae density of *Stylophora pistillata* (n=5-6, ±SE) growing at 5m

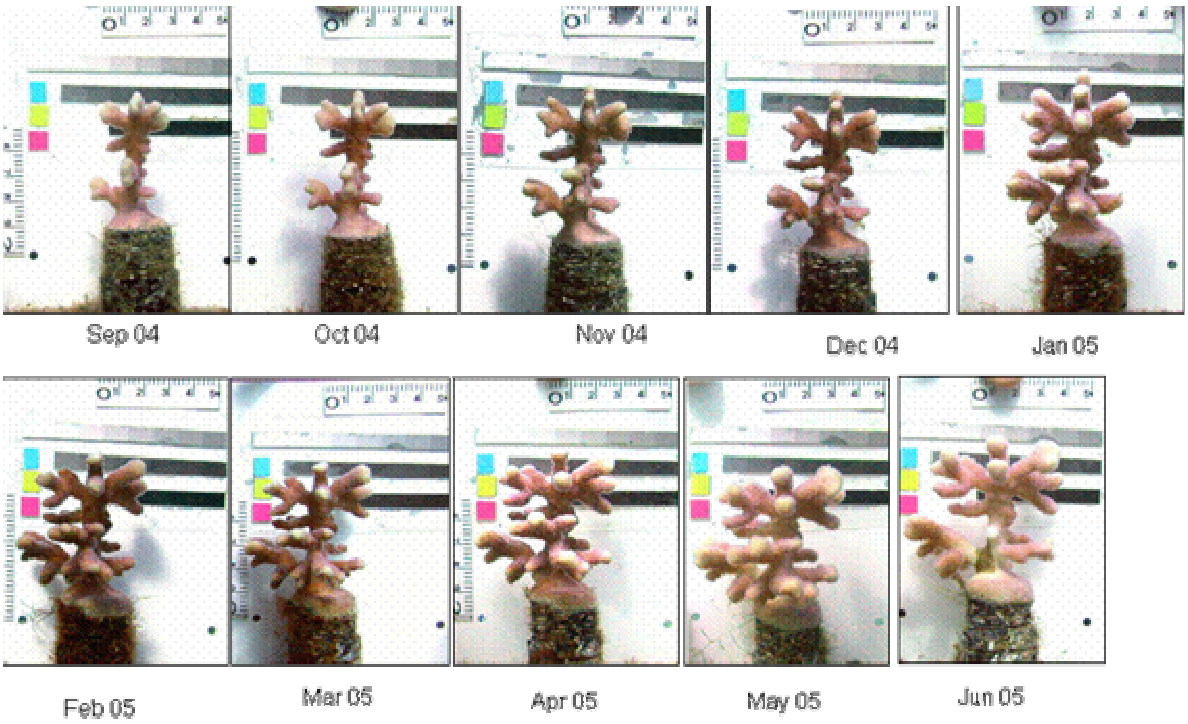

Fig. 6. Photographs following the same branch of *Stylophora pistillata* growing at 5m through out the year.

The cellular mechanism of coral bleaching

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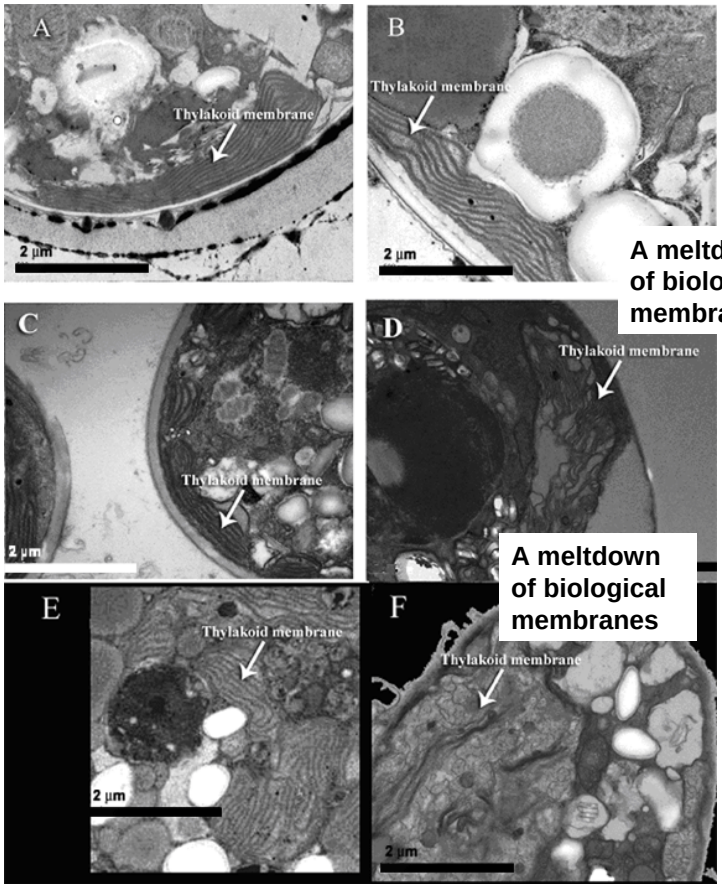
The phenomenon of mass coral bleaching has developed into a major concern because of the coral reef's key ecological role as a major habitat for the most diverse community in the marine realm and its key economic function in numerous economies. Coral bleaching is induced by positive anomalous temperatures in surface waters of 1.5 to 2 ºC. However, not all reefs or corals within a reef are equally susceptible to elevated temperature stress. Here we wish to report that thylakoid membrane (TM) lipid composition in the algal symbiont plays a key role in determining the symbiosis' susceptibility to thermal stress. However, there seems to be no correlation between phylogenetic assignment of the symbiotic algal type and TM lipid composition. In addition, we show that apoptosis, triggered by the production of reactive oxygen species (ROS) in the symbiotic algae (zooxanthellae) that reside within host animal cells, can induce an apoptotic caspase cascade in the host animal that leads to expulsion of the algae and can also lead to death of animal. The bleaching process can be experimentally manipulated by the addition of extracellular ROS and caspase inhibitors. This mechanistic explanation is of major importance in enabling a comprehensive understanding of bleaching on a biochemical and molecular level. Our findings might also reflect on the interpretation of ecological and evolutionary processes that are currently observed in coral reefs world wide.



Theme 2: Diversity, flexibility, stability, physiology of Symbiodinium and the associated ecological ramifications (May 15-17)

Dinoflagellates in the genus *Symbiodinium* are the principal endosymbionts of reef-building corals as well as animal hosts from several other phyla. Understanding the forces that have driven the evolution and distribution of these organisms has the potential to provide important insights into the response of corals to past and present environmental change. This workshop theme aimed to achieve greater synthesis of the large number of studies that have focused on the diversity and specificity within the genus *Symbiodinium*. This resulting discussion focused on the phylogeny, specificity, biology and flexibility of *Symbiodinium* symbioses – particularly where our current understanding is of the ability or not of coral hosts to adapt rapidly to climate based stresses by switching *Symbiodinium* partners. One of the key ambitions of this discussion was to produce a consensus statement that will help guide future research and project the current state of our understanding of the field. This was achieved and appears at the end of this section of the workshop document.

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Participants:

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Goals of workshop component:

The goal of the special discussion was to assess our current state of understanding of *Symbiodinium* symbioses in terms of:

1. Genetic diversity and taxonomy of *Symbiodinium*.
2. The cell biology of *Symbiodinium* symbiosis (initiation, selectivity, recognition)
3. The ecological & physiological benefits of harboring different symbionts.
4. Stability of *Symbiodinium*-host (=holobiont) combinations in time and space.
5. Flexibility by hosts in terms of establishing or swapping genotypes of *Symbiodinium*.

The diversity, specificity and flexibility of *Symbiodinium* symbioses.

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The dinoflagellate symbioses involving reef building corals are amongst the most spectacular associations between animals and photosynthetic organisms. The principal symbionts of corals (as well as organisms from at least five phyla) belong to the genus *Symbiodinium* (Freudenthal 1962). In most cases, *Symbiodinium* spp. live endosymbiotically within host cells with the notable exception of some molluscs, where they are found extracellularly. Within their host invertebrates, *Symbiodinium* spp. photosynthesize at rates comparable to free-living dinoflagellates but pass over 90% of the newly fixed carbon to their hosts. In exchange, *Symbiodinium* receives access to the normally limiting inorganic nitrogen and phosphorous. In the case of symbiotic Scleractinian corals, these dinoflagellate symbionts power metabolic needs and provide the energy required for the precipitation of enormous quantities of calcium carbonate. The precipitated calcium carbonate in turn forms the primary framework of coral reefs, which is habitat for hundreds of thousands of species. These highly productive structures also protect coastlines in many parts of the world and provide subsistence and livelihood for several hundred million people.

Understanding the taxonomy, biology and evolution of *Symbiodinium* is particularly important given their central functional role within coral reefs. Until recently, however, our understanding of these organisms has been limited, primarily due to the cryptic diversity of *Symbiodinium* and the more ecological focus on coral reef studies over the past 50 years. Combined, this situation has left us without a good understanding of how the association between *Symbiodinium* and its hosts is established, or how flexible the association is with respect to its symbiotic partners. Both of these questions have come to the forefront recently within the question of how coral reefs will respond to global climate change. On one hand, the recently discovered diversity within the genus *Symbiodinium* is seen as evidence of enormous flexibility in ecological time scales and as evidence that corals will adapt quickly to climate change. On the other hand, the diversity is being interpreted as evidence that host and symbiont are relatively faithful to each other, and that changes in the partnership only occur at deeper, more evolutionary time scales. Given the importance of the issue and the diversity of opinion within the field, focusing the workshop on this issue is particularly important. In this paper, I will set out some of the background to the issues which will be taken up by the workshop participants in later papers.

The cryptic diversity of Symbiodinium

S*ymbiodinium* was originally described from the symbiont of the jellyfish *Cassiopeia xamachana* by Freudenthal (1962) and is now recognized as a member of the family Gymnodiniaceae (Class Dinophyceae, Order Gymnodiniales; Freudenthal 1962, Trench 1987). Suspicion that *Symbiodinium microadriaticum* was in fact a number of species began with Robert Trench and his students in the late 1970s. Their studies eventually revealed large differences between cultured *Symbiodinium* from different hosts. This included differences in isozymes, morphology and the ability of *Symbiodinium* to establish symbioses with different host species (Schoenberg & Trench 1980 a, b, c). *Symbiodinium* from various hosts also differed in the numbers of condensed DNA bodies (Blank & Trench 1985; Trench & Blank 1987; Blank & Huss 1989), photophysiology (Chang et al. 1983) and in their fatty acids and sterol

composition (Blank & Trench 1985). Analysis of the small subunit ribosomal RNA gene (18S) supported these differences and revealed three major groupings (clades) designated A, B and C (Rowan & Powers, 1991a). Subsequent work revealed that *Symbiodinium* phylogeny included at least eight highly divergent lineages or clades (A through H) on the basis of ribosomal DNA from the nucleus (18S and 28S rDNA) and chloroplast (cpDNA; LaJeunesse 2001; Pawlowski et al. 2001; Santos et al. 2002; Baker 2003; Pochon et al. 2004; LaJeunesse 2005).

These major clades are found in most oceans although Clade C appears to be the most common genetic variety in the Indo-Pacific while Clades A and B share dominance with Clade C in the Caribbean. The majority of coral colonies appear dominated by a single type of *Symbiodinium* (Baker 2003; LaJeunesse 2005). Cnidarian hosts, however, sometimes contain several clades (Rowan and Knowlton 1995; Rowan et al 1997; Loh et al 1997; Lewis and Coffroth 2004; Little et al. 2004). Within colonies, these divergent types of *Symbiodinium* may occupy different microhabitats within a host (Rowan et al 1997) or different depths (Baker et al. 1997).

Investigation of *Symbiodinium* using higher resolution markers such as internal transcribed spacer regions (ITS 2, LaJeunesse 2001; ITS 1, Van Oppen et al. 2001), microsatellites and flanking sequences (Santos et al. 2004), and DNA fingerprinting (Goulet and Coffroth 2003) have subsequently revealed even greater complexity within the rDNA defined clades of the genus *Symbiodinium*. Using ITS 2, LaJeunesse, van Oppen and coworkers have uncovered distinct genotypes that vary with host genera. These patterns indicate that co-evolution of host and symbiont has occurred given that the distribution of these sub-cladal lineages is not random among coral hosts. For example, C15 is distinctive of *Porites* while C3 is characteristic of *Acropora* and other host species across the vast areas of the Pacific Ocean (LaJeunesse et al. 2004). More recent work shows that ITS lineages within the major clades of *Symbiodinium* also vary with depth and are likely to represent functionally distinct genotypes or species of *Symbiodinium* (Iglesias-Prieto et al. 2004; LaJeunesse 2005). As was seen with the major clades of *Symbiodinium* (A – H), cnidarians appear capable of hosting several varieties of ITS genotypes, which may vary according to environmental conditions and host ontogeny. These issues will be discussed further below. Recent work is also indicating that care must be taken with respect to intragenomic variation as regards the interpretation of ITS patterns. In this case, single celled PCR amplifications have revealed that cells may contain ITS copies that classify out as several different clades or ITS genotypes (Van Oppen et al, in press; Gates personal communication). Dominant patterns, however, may still hold although the reality and abundance of multicladal assemblages is now open to some question.

The establishment of coral-Symbiodinium symbioses

Despite its importance, we know very little about how the symbiosis between *Symbiodinium* and its hosts is established. Work by Virginia Weis and others is using perspectives from analogous symbioses like Hydra-Chlorella to develop a better understanding of the cellular events that lead up to the establishment of coral-*Symbiodinium* associations in species such as *Fungia*. In this case, the observation of specific phagocytotic processes that engulf algal cells (e.g. McNeil 1981) and the inhibition of phagosome-lysosome fusion (e.g. Hohman et al 1982) give clues to the complex set of processes that are likely involved in the recognition and incorporation of potential symbionts into coral host cells. Experiments similar to those in Hydra that focused on the cellular events are only in the various earliest stages. A host of other studies are now using molecular tools to investigate how the diversity of associates within corals changes as the association matures. In this regard, there

appears to be some very interesting patterns in which the initial uptake of *Symbiodinium* appears relatively unspecific but which narrows with maturation of the colony (Little et al. 2004; Gomez 2005). This narrowing phase involves the eventual dominance of 1-2 genotypes, which may or may not involve the retention of some of the original diversity at background levels within the tissues of the adult corals. It is clear we need to understand more about these steps in the establishment of a mature symbiosis. Questions such as to whether there is a functional significance to having particular varieties of *Symbiodinium* in the early stages of a symbiosis need answering.

The establishment of the association between *Symbiodinium* and coral hosts might be analogous to a potential guest hoping to enter a house (Figure 1). To get in the front door, the guest might need to possess a key, one which is commonly available to the particular group (in this case, the genus *Symbiodinium*). This might be the right lectins on their membrane surface that result in a particle or alga being exocytosed. Once through the front door, however, the group of guests only have access to the lobby of the house. To go any further, the guests need to possess a second key. In actuality, this might be the ability to avoid lysosomal attack by having the right surface molecules to avoid recognition as foreign particle. Through this door, only a select few guests can enter the corridor. But as before, the guests require a third key. This might be in reality the ability to integrate metabolically with the host, allowing the passage of substrates through the double membrane in which the alga now lives. Through this door, a small subset of the original guests will pass into, say, the kitchen. At this point, integration of host and symbiont via cell signalling processes prevent *Symbiodinium* from dividing out of step with the host cell might be the critical next step. And so on. Under this model, the narrowing of diversity might be due to the relative proliferation of the appropriate type of *Symbiodinium* (B in Figure 1) over others, given it posses all the "keys". Those that gain access to only the lobby on the other hand, may be left in a "semi-symbiosis" within this space. This may explain the recent observation of so-called "moonlighting" *Symbiodinium* in many hosts.

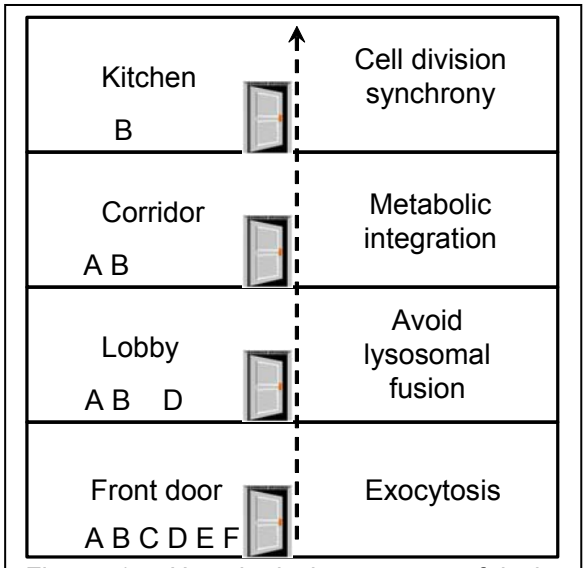

Figure 1. Hypothetical sequence of locks and keys involved in the establishment of a symbiosis between *Symbiodinium* and a coral host. Letters are akin to genetic varieties of *Symbiodinium*.

These cellular events are likely to be highly selective, as one would expect in any cellular process in which one cell goes to live within another. Studies that have followed the genotypes of *Symbiodinium* within corals over time (in and out of bleaching events) have revealed enormous stability within ecological time scales (Gomez 2005; Stat 2005). Experimental manipulations have revealed in a few instances (e.g. Baker 2001) that the relative proportions of different genotypes of *Symbiodinium* may change. However, the definitive proof that new combinations are indeed novel remains to be established (Hoegh-Guldberg et al. 2002).

Flexibility of coral-Symbiodinium assemblages over short time scales?

The mass bleaching of corals is one of the most dramatic changes to take place on a coral reef. In these events, the symbiotic association breaks down as conditions place the association under stress. In the case of recent global episodes, mass bleaching is caused by warmer than normal sea temperatures (Hoegh-Guldberg 1999). Buddemeier and Fautin (1993) proposed the interesting idea that bleaching may represent a strategy by which hosts may exchange their symbionts for ones that have higher thermal tolerances. As will be discussed in this workshop, the idea has largely been shown not to hold true, especially if the case is restricted to case of "evolutionary switching" as opposed to "shuffling". In the former case, coral bleaching has never been shown to result in a completely novel symbiotic association, as would be necessary if an association were to "evolve" suitably new thermal tolerances to deal with escalating sea temperatures due to climate change. Shuffling, the case where changes occur in the relative proportions of different *Symbiodinium* genotypes within multi-cladal hosts, does not lead to truly novel symbioses and hence doesn't lead to required rapid changes in the thermal tolerances needed to keep up with the current high pace of climate change.

The current debate requires resolution given its importance to how coral reefs may fare under rapid climate change. From recent exchanges (e.g. Baker 2002 versus Hoegh-Guldberg et al. 2002), it is clear that there is need to clarify terms that are often used loosely yet the specific meaning of which is critical to any resolution of the debate. It is also clear that time scales are important. In this regard, evidence of evolutionary switching that occurs rarely over long time frames (at least 10,000's of years if not 100,000s of years, Stat 2005) should not be used to support the idea that corals will be able to change their thermal tolerances in the short intervals typified by bleaching events and rapid climate change. That is, the observation that a coral has a genotype of Symbiodinium (Clade E) which is normally found in Foraminifera (Rodriguez-Lanetty et al. 2000) is not proof that evolutionary switching occurs with enough frequency to cause the required upward changes in thermal tolerances required to allow a reef to remain robust under rapid climate change. One has only to calculate the required change in the thermal tolerance for corals and their Symbiodinium (between 0.2–1.0^{o}C per decade, Donner et al 2005) to keep pace with even mild rates of climate change to appreciate the challenge populations of corals and their *Symbiodinium* face over this present century.

This workshop took up many of these issues and has provided a series of papers that review the exciting new developments in the field. In doing this, the workshop has indicated where we should be putting greater emphasis in future studies. Most importantly, it has provided a consensus statement at the end of this section, which summarises the current understanding of many key issues. The consensus that was achieved in Mexico provides a very useful clarification and set of common terms by which to discuss complex issues such as the flexibility of the symbiosis between corals and *Symbiodinium*. This represents a great basis from which to start to address the critically important issues of the adaptability of reef-building corals (or not) to climate change.

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Functional diversity of Symbiodinium: the evidence and the history.

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Over the last 10 years we have witnessed a dramatic shift in the paradigms addressed by scientists working on the biology of algal invertebrate symbioses. The realization that not all "zooxanthellae" were created equal has had major implications for our understanding of the biology, ecology and evolution of coral reefs. Contrasting to the early realization that invertebrates harboring symbiotic phototrophic dinoflagellates form a diverse assemblage, the assessment of the diversity within the genus *Symbiodinium* has been a difficult task involving the establishment of axenic mono-algal cultures, the taxonomic description of different *Symbiodinium* species and the development of different genetic markers (Fig 1). As a result of progress in these areas, we have supplanted the notion of a pandemic zooxanthellae, for our current perception of the genus as a highly diverse and specific group. Although progress in this field is the result of the work of many scientists, the pioneering work of Bob Trench and his co-workers was instrumental for the development of our current understanding.

Assessing phenotypic variability in Symbiodinium in cultures

In the absence of suitable genetic markers, the assessment of phenotypic variability within the genus *Symbiodinium* was limited for a number of years to the use of axenic mono-algal cultures. The successful establishment of these cultures was crucial development. Comparisons of different culture's growth under identical conditions in defined media permitted the description of the phenotypic plasticity of symbionts isolated from different sources. By the early part of the 1980s the accumulation of behavioral, physiological, biochemical and structural evidence lead to the suggestion that symbiotic dinoflagellates isolated from different animal sources constitute dis-continuous genetic entities (Schoenberg & Trench 1980). Based on detailed ultrastructural analyses of cultured *Symbiodinium,* the first taxonomic descriptions of species within the genus were published. Today only six species of *Symbiodinium* have been formally described. Considering the importance of algal photosynthesis to the well being of the intact associations, it is not surprising that significant efforts were devoted to describe the variability of the photosynthetic responses of cultured *Symbiodinium* isolated from different animal hosts (Iglesias-Prieto & Trench 1997). Collectively, these studies show that different algal symbionts have significant differences in their photoacclimatory abilities and that these differences probably represent adaptation to different light climates. Although the physiological diversity of

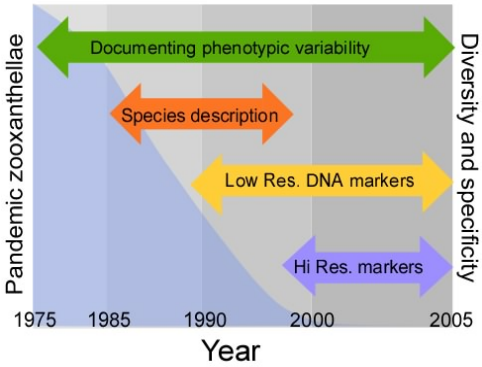

Fig 1. Major developments in the field.

the genus was firmly established by the end of the 1980s, the lack of suitable genetic markers hindered the investigation of the ecological and evolutionary implications of such diversity in the reef.

Linking genetic and physiological diversity in the field

This field of research experienced a dramatic change after the introduction of reliable genetic markers. Restriction analyses of the ssuRNA gene permitted the identification of several clades of uncertain taxonomic value (Rowan & Powers 1991) which allowed us to initiate a global survey of the genetic diversity of the genus. One of the most important limitations of the use of genetic analyses based on the ssuRNA gene is its lack of resolution. This technique fails, for example, to distinguish between two *Symbiodinium* types that have been identified by traditional techniques as belonging to different species (Rowan & Powers 1991). Early attempts to correlate clade designation and physiological performance were based the distribution of algal types along environmental gradients. Unfortunately, the assignment of physiological properties to each clade was premature and not based on empirical physiological data. This approach resulted in unsubstantiated generalizations regarding the functionality of the different clades. Different cultured *Symbiodinium* in clade "A" exhibit a wide range of photoacclimatory responses comparable to those observed among algae in different clades. The use of high resolution genetic markers such as the ITS2 region has successfully identify all the described species in the genus, and has the potential to describe genetic variation of the genus *Symbiodinium* in the field at a scale close to the species level (LaJeunesse 2001). The combined use of high resolution genetic markers and non-invasive techniques such as Pulse Amplitude Modulated (PAM) flourometry allow us to measure directly in the filed the physiological performance of different algal genotypes (Iglesias-Prieto et al. 2004). We showed that the vertical distribution of the two dominant scleractinians in the Eastern Pacific along an irradiance gradient could be explained exclusively on the basis of the photosynthetic performance of their respective specific symbionts. This type of information suggests that for ecological and evolutionary purposes, the unit of selection is the symbiotic phenotype (holosymbiont). It has been suggested that host can respond to variations in the environment by changing the composition of their symbionts (Buddemeier & Fautin 1993). Testing this possibility requires the assessment of the physiological and ecological performance of the holosymbiont.

Conclusions and future directions

Currently, one of the most pressing questions that we face is how reef coral will respond to the environmental challenges imposed by climate change, in particular what role will be played by *Symbiodinium* in determining the limits of acclimation and adaptation of their hosts. In principle there is enough functional variability within the genus to accommodate potential hosts to very different environments. In this context, it is imperative that we determine how specificity controls host range in nature. Addressing this question requires the simultaneous utilization of genetic and physiological techniques.

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Diversity and specificity of *Symbiodinium*

William K. Fitt

This talk was designed to ask questions (that we don't know all the answers to), to give partial answers that consist of real data, and then speculate wildly about the rest of the answer.

What are the major patterns of diversity of algal symbionts?

There is an increased diversity of Symbiodinium in shallow water vs. deep water, in marine habitats closer to the equator, in hosts closer to shore, and in the Caribbean vs. Indo-Pacific (e.g. LaJeunesse 2002, et al. 2003. 2004a,b). Symbionts that are relatively rare at some reefs may be extremely common on other reefs.

We hypothesize that high-diversity Symbiodinium indicates reefs that are under more stress than low-diversity reefs.

What are the steps in establishing specificity in the endosymbioses?

There are at least three studies documenting uptake of Symbiodinium from the water column that suggests that there is little specificity for juveniles of broadcast spawners – they take up what is available and sort it out later (Coffroth et al. 2001, Thornhill et al. 2006, Cabrera et al. 2006).

We hypothesize that there is no specificity in uptake (phagocytosis) of Symbiodinium, and that all symbiotic cnidarians can take up all types of Symbiodinium throughout their lives!

What is the evidence for, and significance of, free-living Symbiodinium?

Free-living Symbiodinium exist, and infect aposymbiotic hosts in nature and in the laboratory. We don't know where they are coming from, nor what they are doing.

We hypothesize that most Symbiodinium are free-living, and that only a small subset inhabits invertebrates.

What are the physiological correlates of having different types of Symbiodinium?

Since Symbiodinium ranges in size from about 6 to 12 um, the volume has a 4x range. Whether one looks at scyphistomae or giant clams, one can see a whole range of potential differences in photosynthesis and respiration rates, calcification rate, etc, as well as a way to competitively displace one symbiont by another.

We hypothesize that the host digestive cell acts as a "culture-tube" and plays a passive role, but specificity resides in the host-cell properties.

What is the evidence that hosts are flexible in their associations with Symbiodinium?

We hypothesize that ALL host species harbor several (dozens to hundreds?) of types of Symbiodinium at the same time. Most hosts establish a relationship whereby one type of Symbiodinium comprises over 95% of the cells.

What is the evidence that "stressed" hosts can "pick-up" or "change" complements of Symbiodinium?

We hypothesize that during abnormally high temperature type D1a can become "life-jackets" for the host. Under normal conditions D1a is not optimal and the host must acquire an optimal zooxanthellae type to survive.

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Symbiodinium systematics: molecular markers and the techniques appropriate for eco-evolutionary investigations.

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The analysis and characterization of *Symbiodinium* diversity is at a crossroads. A total of eight divergent clades are recognized and their taxonomy is largely in agreement. However, the classification of numerous genetic types/strains/sub-clades within each of these main lineages is in disarray. Much of the confusion resides in the use of different genes and/or certain techniques that do not allow for cross-comparison between findings from different research laboratories. Progress in the physiological, ecological and biogeographic research on the coral-algal symbioses requires a consensus on sub-cladal taxonomy (and the methods used to identify it). The taxonomical significance of within-clade diversity must first be addressed. Viewed from one extreme, these "types" are merely sequence variants within a single metapopulation (clade=species). At the other extreme, each variant appears to represent an ecologically distinctive "species" on an independent evolutionary trajectory. Interpretation of the true significance of this genetic diversity appears to hinge on the techniques employed to measure it.

As for most microorganisms, molecular techniques are required in examining, assessing, and identifying the diversity of coral endosymbionts. Commonly referred to as zooxanthellae, dinoflagellates in the genus *Symbiodinium* were once collectively assigned to a single panmictic species. Results from morphological, biochemical, behavioral, and physiological studies by Trench and colleagues in the 1970's and 1980's began to dispel this established belief. However, it was the publication of the first ribosomal DNA sequences by Rowan and Powers (1991) that finally convinced many of, or made them aware of, the potential diversity that existed within this symbiont group. Since then, numerous studies involving comparisons of DNA sequence diversity have been published. Desire for increased genetic resolution resulted in the analysis of more rapidly evolving genes. Starting with the conservative nuclear ribosomal SSU 18S, analysis shifted to the LSU and then the internal transcribed spacer regions (ITS). The recent application of comparing microsatellite loci (and DNA fingerprinting) has initiated population level genetic investigations of these microorganisms.

In efforts to increase the number of samples sequenced, speed the process, and reduce costs, single strand conformational polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) were employed. Both allow the resolution of single base pair differences in the DNA region of interest.. Each method produces repeatable fingerprint patterns that are consistently observed from sample to sample. Both techniques, however have drawbacks. For SSCP, the quality of the fingerprint image is relatively poor making the characterization and subsequent identification among numerous unique fingerprints difficult. The direct excision of bands from SSCP gels, followed by their re-amplification and direct sequencing, is not possible. Instead, left over PCR products from the initial amplification must be shotgun cloned and sequenced. Direct sequencing, especially of the ITS region, is often problematical. This is most likely due to high levels of intragenomic variants that are

present throughout the ribosomal array of many Symbiodinium. The presence of a mixed template in a sequencing reaction leads to nonsensical sequences. The solution is to clone from these PCR products and then sequence. The major problem with shotgun cloning is that a cloned gene copy may or may not be a representative marker for the organism. Typically, one or two variants are the most common in a genome and are presumably responsible for diagnostic SSCP /DGGE fingerprints. If they cannot be identified, numerous cloned copies must be sequenced in order to properly characterize the extent of this intragenomic diversity. This method requires too much time and money to be practical. In contrast, DGGE produces highly repeatable and clear fingerprint profiles of which the most diagnostic bands are easily excised, re-amplified, and directly sequenced. The main drawbacks of this technique are; 1) the required investment of special electrophoresis equipment and 2) time required to run each gel.

The application of different techniques targeting different DNA regions by so many different research labs has led to confusion about the perceived genetic diversity of Symbiodinium and its ecological significance. Work accomplished by one lab is not readily assessable by others wishing to incorporate it into their own publications. Uncertainty with evaluating and integrating previous work has been especially problematical when describing *Symbiodinium* diversity within each clade. This lack of consistency has stalled progress toward a fuller understanding of symbiont biogeography, host-symbiont specificity, and differences in physiology. It would seem that effort is needed in establishing a consistent technique that reliably distinguishes between ecologically distinctive forms.

Standardizing a molecular taxonomy for Symbiodinium beyond the "clade" level would dramatically improve the scientific progress of our field. Fundamental to any endeavor in ecological or comparative physiological research is the accurate identification of the organisms under study. A candidate approach using denaturing gradient gel electrophoresis (DGGE) of the ITS rDNA does provide a consistent identification of genetically similar yet ecologically and biogeographically distinct Symbiodinium. Work is still required to "ground-truth" whether or not these 'types' signify genetically isolated species. Because the ribosomal array contains a high degree of intragenomic variability, careful analysis and interpretation of this variability is necessary. The development of additional nuclear, mitochondrial, and chloroplast markers will offer direct and indirect testing of the ecological and evolutionary significance of these sub-cladal "types."

Coral bleaching as an exaptation that can promote rapid and beneficial change in algal symbiont communities

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Semantic confusion

The Adaptive Bleaching Hypothesis (ABH, Buddemeier and Fautin 1993), now over a dozen years old, has a name that is both powerful and dangerous. Powerful, because most readers intuitively understand the general concept from the name alone (resulting in the ready incorporation of the term into the research lexicon), but dangerous because this intuitive understanding is often accompanied by secondary meaning in the words used.

Much debate – published or otherwise – over the ABH has centered over the use of the word "adaptive" (Buddemeier et al. 2004), which most biologists interpret as implying a process acting over evolutionary time that favors certain heritable traits over others, leading to a directional change in the relative abundance of genes in a population over time. This perspective has led to criticism of the ABH on the grounds that bleaching (and subsequent recovery, occurring over timescales of weeks to months) provides insufficient time for evolution to act (*e.g.*, Hoegh-Guldberg et al. 2002). However, these arguments have generally failed to address the real questions of interest, which are less concerned with whether bleaching is "adaptive" (or not), and more concerned with whether it promotes change in symbiont communities, and what the effects of these changes might be.

Critics of the ABH maintain that bleaching evolved to remove damaged (and therefore harmful) symbionts as part of the immune response. However, traits can be adaptive for multiple reasons, not all of which need to contribute equally to the selection pressure on a trait over the course of its evolution. Bleaching as an immune response can still have added adaptive value if it has the side effect of promoting rapid symbiont community change that benefits the coral host. Perhaps when viewed in this way (as an exaptation), the ideas encapsulated by the ABH might be less contentious.

Sublethal bleaching and the ABH

Most of the interest surrounding the ABH has little to do with evolution, and everything to do with ecology. Does bleaching result in symbiont change within an individual colony frequently enough to be ecologically meaningful? Or is bleaching principally an agent of natural selection, causing differential mortality within a population or community of corals determined to some degree by the symbiont communities they contain? These two questions may be fundamentally linked if periodic sublethal bleaching expels certain symbiont types over others, and subsequent recovery involves the stochastic acquisition of novel symbionts from the environment. If this is the case, mechanisms of symbiont regulation, including low-

level seasonal bleaching (Stimson 1997, Brown et al. 1999, Fagoonee et al. 1999, Fitt et al. 2000), may introduce critical background variation into symbiont communities that ultimately provide the backdrop against which shifts in symbiont communities in response to severe bleaching and mortality events (Baker et al. 2004) are able to occur (Fig. 1).

Acquisition of symbionts from environmental sources

Figure 1 emphasizes the importance of cryptic or "minor" symbionts in determining the responses of corals to bleaching, and assumes that most (perhaps all) coral species are able to acquire symbionts from the environment in the adult phase, as is the case in certain non-scleractinian anthozoans. Probability theory supports this assumption: if symbiont diversity is solely determined at the larval stage (as a subset of maternal and/or environmental symbionts), and if adult colonies only possess mechanisms for losing this diversity (through bleaching), then symbiont diversity would inevitably be purged from adult colonies over time. Consequently, large coral colonies, as well as coral species that maternally transfer symbionts from generation to generation, would become monotypic in their symbionts over time. Molecular survey data indicate this is not the case, suggesting that adult colonies can and do acquire symbionts from environmental sources.

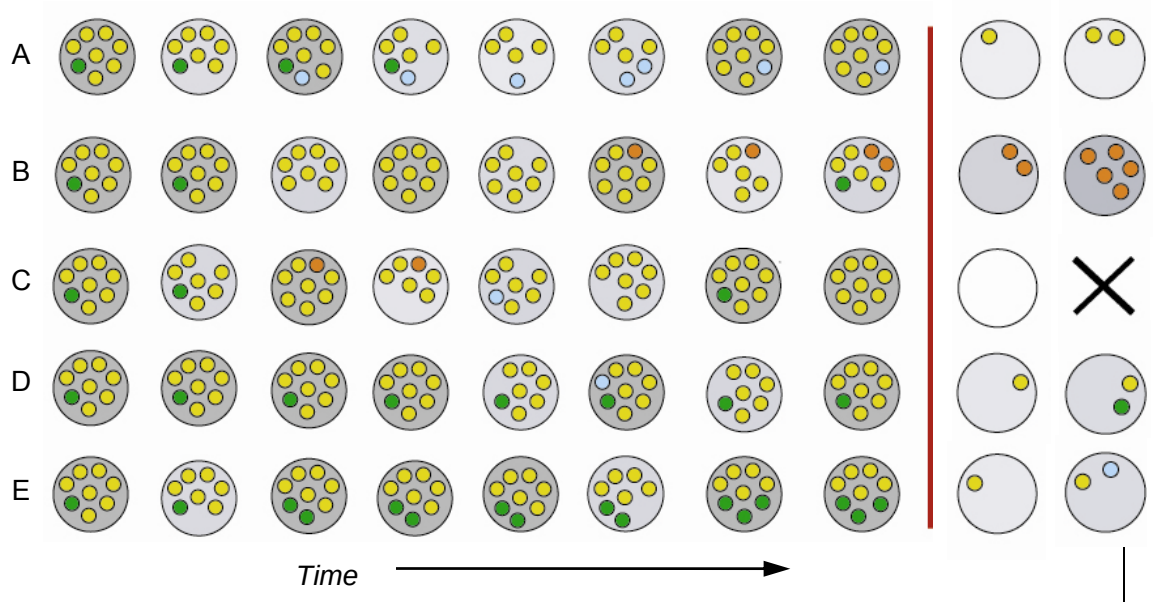

Fig. 1. Conceptual model illustrating how low-level bleaching and recovery of coral colonies maintains critical background variation in algal symbiont communities. In this model, five healthy conspecific coral colonies (A-E, larger circles) start with identical symbiont types (smaller circles, with different symbionts represented in different colours). These colonies experience different levels of sublethal bleaching over time (varying shades of grey). After eight time steps, all colonies are dominated by the same symbiont type (yellow) but contain different minor symbiont types. A severe bleaching event (red vertical line) leads to dramatic loss of symbionts in all colonies, followed by recovery (increasing numbers of symbionts) or mortality (X). In this model, orange symbionts are resistant to bleaching, leading to a shift in colony B to favor these symbionts following severe bleaching. This change in dominance is a result of variation in minor symbionts existing prior to the event.

Future questions and research directions

Current debate centers on the timescales over which symbiont change can occur, the extent to which symbiont change involves endogenous vs. exogenous sources, and the degree of bleaching and/or mortality of coral hosts that might be necessary for changes to be detectable. Fortunately, all of these questions are tractable from a research perspective. However, there has been surprisingly little research in these areas over the last dozen years, with active research focusing instead on the diversity and distribution of *Symbiodinium* in different host species. In order to make real progress in this field, these questions should be moved to the top of the agenda.

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Modeling the Adaptive Bleaching Hypothesis

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The adaptive bleaching hypothesis asserts that coral bleaching (the loss of the symbiotic algae that give corals their colour) may be an adaptive mechanism. By providing an opportunity for recombining hosts with alternative algal types that might be better adapted to altered circumstances, bleaching may result in improved resistance to increasing stress. Once thought to be a single taxon, recent studies have demonstrated that the symbiotic dinoflagellates which inhabit the coral body cells have significant taxonomic diversity.

The ABH was first postulated by Buddemeier and Fautin in 1993. Shortly thereafter, Ware, Fautin, and Buddemeier (1996) published a paper describing a simulation of the ABH which provided some interesting results. More importantly, the 1996 paper made explicit the assumptions of the ABH, some of which were implicit in the original paper.

Since the publication of these two papers, the ABH has been the center of one of the most intense controversies in coral reef science since Darwin's publication of his coral reef hypothesis.

Clearly, if true, the ABH would imply that corals had some sort of 'hedge' against global warming. By shifting their symbionts to ones with higher temperature resistance, corals might be less sensitive to long-term temperature increases than previously thought. I summarized this thought with the following limerick:

An elderly coral was teaching
The younger corals 'bout bleaching:
A word to the wise:
As temperatures rise,
Change the zooxs that you all are hosting!

In addition to presenting simulation results related to the ABH from the original paper that appeared in Ecological Modeling and from a presentation at the SICB in Boston some years later, I discussed the process of developing mathematical models of biological concepts and the advantages which accrue from such development.

From my viewpoint, the primary advantages of developing mathematical and computer based models are that: (1) they focus and clarify what might have been fairly vague thoughts during the formulation of a concept; (2) assumptions that may have been implicit in the formulation phase, must now be made explicit; and (3) despite the fact that nothing comes out of a computer model that was not put in, some results may not have been anticipated.

One of the interesting aspects of the simulation approach is that the climate model used to develop the results presented is not the more common, physics based formulation. General Circulation Models (GCMs) attempt to model the physics of the climate by dividing the atmosphere (and sometimes a portion of the oceans) into blocks and follow the flow of energy into and out of each block. The resulting models are extremely complex and, depending on the granularity with which the atmosphere

is modeled, require massive computer resources; thereby precluding multiple simulations. In contrast, the basic temperature model that I have developed is based on both long-term (centuries) and short-term (years) temperature observations. The resulting model is extremely simple (by comparison) and permits hundreds to thousands of repeats with differing initial conditions.

Several of the common fallacies and observations associated with computer-based models are emphasized. These include:

- Complexity = Accuracy. The fact that GCMs are based on sound physical principles does not equate with precise emulation of the Earth's temperature evolution. For example, GCMs would not demonstrate the Little Ice Age, whereas the simpler model does occasionally show LIA type behavior. In addition, GCMs typically contain a large number of parameters whose true values are not accurately known.

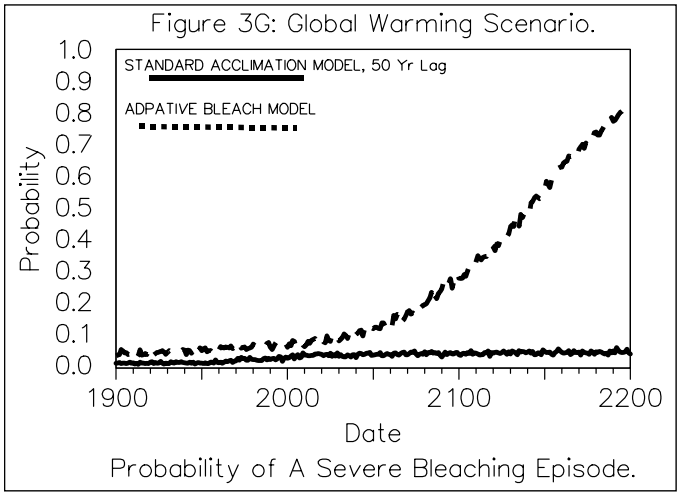
- Precision = Accuracy. Computer models may provide results to 6 decimal places. This in no way implies that the results are accurate.

- Validation and verification of computer models are things that engineers do. Science does not require V&V (that is, the more intelligent the programmer, the less likely they are to check (verify) their computer programs).

Using temperature as a surrogate for all aspects of global climate change and bleaching as a surrogate for all responses, I compare the response of corals under the ABH and under a gradual acclimation model.

The simulation results show that, if the ABH is valid, reef corals may acclimate rapidly and resist climate change for a substantial period, perhaps another 25 to 50 years. However, ultimately the rapid changes expected as a result of anthropogenic perturbation of global climate, augmented by the hundreds of point sources of pollution on or near reefs, may cause many coral reefs to change their community structure precipitously.

The figure below, Figure 3G, allows comparison of the probability of severe bleaching events for corals which acclimate with a time lag of 50 years versus "ABH corals" under a global warming scenario of 1.2 Co per century. As can be seen, the ABH corals appear to be doing fairly well until approximately 2030 to 2050 at which time probabilities of severe bleaching events increase precipitously leading to the ultimate demise of the coral reef ecosystem. If, on the other hand, corals and/or their algal symbionts can acclimate with even a 50 year time lag, which I consider highly unlikely, coral reef survival is possible. Ultimately, if sea surface temperatures continue to increase, acclimation potential will be exceeded and the reef system will collapse. (Probabilities are estimated from 1000 runs of a simplified climate model.)



In summarizing, it is extremely important to note that current global climate change should NOT be interpreted as being of benefit to coral reefs. The ABH suggests that bleaching may allow corals to acquire symbionts that are more resistant to increased temperatures than otherwise. This does not mean the holobiont (combination of coral and symbiont) is better off than before the perturbation that caused the bleaching. In fact, the new holobiont must be at some disadvantage in the absence of perturbation or a shift to a new symbiont would have occurred without the perturbation.

Dynamics of cnidarian-*Symbiodinium* symbioses: Thoughts on flexibility, stability and ontogeny of the symbiosis

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The endosymbiosis of the dinoflagellate *Symbiodinium* spp. with cnidarians is one of the most striking and ecologically important relationships in the marine environment, having profound effects on the hosts' physiology and ecology. Although the symbiosis had been regarded as an almost invariant and unchanging feature, it is now recognized that symbionts within a single host are both diverse and dynamic, changing ontogenetically and in response to environmental conditions. Some Caribbean scleractinians harbor multiple *Symbiodinium* taxa either simultaneously within a single host colony or across reef habitats, while in other host taxa (i.e., octocoral) the symbiont complement is less diverse and often does not vary with changes in environmental conditions. Thus, cnidarian-*Symbiodinium* symbioses present a continuum in both *Symbiodinium* diversity and in selectivity in the pairing of host and symbiont.

Given this potential for variability, it is important to determine how *Symbiodinium* diversity in adult cnidarians is maintained and the factors that control the diversity and distribution of the different taxa of *Symbiodinium* among their cnidarian hosts. To address the effects of global warming on reefs and to understand phenomena such as bleaching, it is necessary to determine if the established *Symbiodinium* complement is fixed or able to undergo further change. Variation in small subunit ribosomal rDNA (ssrDNA), large subunit ribosomal DNA of the chloroplast (cp-23S-DNA) and a series of microsatellite loci were used to determine patterns of *Symbiodinium* diversity over spatial and temporal scales and the flexibility (and stability) of the symbiosis under normal and stressful environmental conditions.

Variation/diversity on a spatial scale

Symbiodinium diversity within the host species *Porites divaricata, Gorgonia ventalina, Briareum asbestinum* and *Pseudopterogorgia elisabethae* was examined in the Florida Keys and the Bahamas. *Symbiodinium* clades within the scleractinian *P. divaricata* varied across the Florida Keys, with *Symbiodinium* clade A detected more frequently in the middle keys than in the upper or lower keys. Within *B. asbestinium, Symbiodinium* cp 23S rDNA type also varied with location with type B184 more common in the lower keys. *Symbiodinium* diversity was also detected at the population level. For example, within *G. ventalina*, variation in microsatellite allele frequencies was detected across the Florida Keys. High levels of genetic structure was also found among symbiont populations isolated from P*. elisabethae* host populations in the Bahamas (Santos et al 2003). On a single reef the majority of host colonies harbored the same symbiont microsatellite genotype but that genotype differed between reefs. It is not known if this distribution of zooxanthellae reflects local availability of the symbionts and if that variability is generated by restricted dispersal or microhabitat differences and niche partitioning among the symbionts.

Variation/ flexibility on a temporal scale

Symbiodinium diversity was also examined on a temporal scale by following the zooxanthella complement in octocorals over time. Symbiont identity within individual

colonies of *Plexaura kuna* was followed for up to 10 years over multiple reefs using multiloci DNA fingerprinting and ssrDNA. No change in symbiont complement was observed (Goulet and Coffroth 1997, 2003ab). *Symbiodinium* abundance, chlorophyll content and genotype within *B. asbestinium* colonies were monitored over a year. Cell densities and chlorophyll *a* content varied, but no significant seasonal variation was observed in the *Symbiodinium* cp-23S-rDNA genotype, suggesting that symbiont pairing in this host species is stable.

Flexibility in algal taxon in early ontogeny

Flexibility in the symbiosis occurs across ontogeny in some host taxa. In primary polyps of many octocorals, initial acquisition of symbionts is non-selective (Coffroth et al 2001). Primary polyps placed over a range of habitats acquire multiple *Symbiodinium* clades within the first 3 months and then over time these symbiotic taxa are winnowed to the single clade found within the adult host. Although the mechanism that leads to the establishment of the establishment of a single clade is not known, field and laboratory experiments have demonstrated that the final adult complement is not dependent on which taxon initially colonizes the host or on early survivorship of the polyps.

Flexibility/stability in response to stress

Among octocorals, the *Symbiodinium* complement within adult hosts does not appear to vary under normal environmental conditions (time and space). However, what happens when the coral is more severely stressed such as in a disease or bleaching event? The symbiont complement within healthy *G. ventalina* and those infected with the fungal pathogen, *Aspergillus sydowii* was monitored in the field and in the lab where the disease was induced. In all cases there was no change in *Symbiodinium* cp-23S rDNA**.** When colonies of *B. asbestinium* were induced to bleach experimentally, low levels of zooxanthellae were detected and these often differed from the original dominant symbiont type. This suggests that cryptic populations may be important in dealing with stress. Bleached colonies of *B. asbestinium* were also exposed to cultures of an isoclonal line of *Symbiodinium* that had a rare cp23S-rDNA allele. Subsequent analysis of cp23S-rDNA of *Symbiodinium* from these *B. asbestinium* colonies recovered the rare allele, confirming for the first time that adult corals retain the ability to acquire new symbionts from the environment (Lewis and Coffroth 2004).

Conclusions and thoughts

These data suggest that within octocorals the typical adult zooxanthella population is established early in ontogeny and does not change under moderate environmental fluctuations (seasonally or disease). When the coral is more severely stressed such as in a bleaching event, there appears to be a sequential loss of zooxanthellae. If the coral survives, the repopulating algae can be from a residual population and/or from exogenous sources. Surveys of zooxanthellae diversity across the Florida Keys and Bahamas established *Symbiodinium* variation within a host species at the population level. These data raise other questions such as what is the mechanism that leads to the establishment of one *Symbiodinium* taxon in a host? Can a new symbiont colonize a host without bleaching? Do patterns of host-alga selectivity reflect species/taxon specific interactions between host and alga, or is *Symbiodinium* community dynamics a function of the suitability of the host habitat to the algal symbiont, or do the dynamics of the zooxanthellae reflect stochastic processes of dispersal, colonization and population expansion within hosts? What happens to these patterns if the established symbiosis is perturbed, and finally, how do these processes vary among host taxa? Knowledge of how *Symbiodinium* diversity within scleractinians and octocorals is established (via initial infection, replacement and/or competition over time) and how flexible these symbioses

are in response to environmental perturbations is essential to understanding host and symbiont distribution patterns and will aid in identifying the processes that are critical in maintaining a viable symbiosis.

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Can the highly variable ITS2-region uncover ecologically relevant patterns in the distribution and persistence of *Symbiodinium* sp. in Pocilloporid corals?

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Coral reefs and stress

The persistence of coral reefs is determined by the finely regulated symbiosis between the coral host and symbiotic algae (*Symbiodinium sp*), where each component determines the success of the complex. Environmental disturbances, such as global climate change, can disturb the finely regulated balance between coral and algae, whereupon the symbionts are expelled from the host tissue (becoming white or bleached) and an entire colony may die (Lesser, 1997; Fitt and Warner, 1995). In addition to large scale bleaching, other localised sources of damage to reefs such as pollution, nutrient run-off and over-fishing, have resulted major damage to coral reefs around the world. Many believe that coral reefs now face catastrophic decline as mass bleaching events and other stresses increase in severity and frequency over the next 30 years (Hoegh-Guldberg, 1999).

The response of corals to bleaching has been found to vary among coral genera and geographic location, indicating a possible role for both the host and symbiont in determining the stress-response (Marshall and Baird, 2000; Brown, 1997). Symbionts have been shown to have variable physiologies, and "same host-different symbiont" combinations may therefore have alternate tolerance limits in accordance with environmental parameters (Baker, 2003, Banaszak et al, 2000; Iglesias-Prieto and Trench, 1997a,b; Buddemeier and Fautin, 1993). More importantly, bleaching tolerant hosts are found in areas where the majority of the population is highly affected (Edmunds, 1994). Unfortunately, very little information is available as to how or why these individuals resist or cope with environmental stress better than those that die.

Genetic variability

The use of molecular techniques has uncovered an enormous diversity of symbionts (LaJeunesse 2001, 2002; Baker et al, 1997; Rowan and Powers, 1991) Here, the use of the highly variable ITS2 DNA region to detect variability in host-symbiont combinations on a local scale was tested to assess whether the huge variability has a physiological function that can be related to the ecology of the symbionts within their specific host species. Given the crucial role that symbiont availability and host-specificity plays in determining what combinations of host and symbiont will be successful, three major goals were to (a) determine if multiple host-symbiont combinations are possible within a single host and whether these are determined by local environmental gradients; and (b) whether these combinations can be adopted by all individuals at any time, and finally (c) if these associations are flexible over time and under altered conditions.

Flexibility

Three ubiquitous species of corals, viz. *Stylophora pistillata*, *Pocillopora damicornis* and *Seriatopora hystrix*, were subjected to a broad sampling regime at multiple depths on two locations around Heron Island (Great Barrier Reef, GBR). Pocilloporid corals in this geographic location are generally reported to host Clade C symbionts, and PCR and DGGE of the ITS2-region were selected to study the intra-cladal variability of symbionts. Individual host colonies of both *S. pistillata* and *P. damicornis* formed associations with multiple symbiont types, and within these species ITS2-types exhibit a marked relation with depth. Each host species has its own community of symbionts, in which each symbiont occupies a specific niche.

Additionally, to determine whether established host-symbiont associations were flexible, a large-scale transplant experiment was established to monitor symbiont populations over a period of two years. Preliminary results from the transplant experiment also indicate that established host-symbiont combinations are not necessarily fixed and may vary over time depending on the stress placed upon them. This study forms part of my PhD research, and the transplants are still in the field for continued collections.

Conclusions and future directions

Most studies to date have focused on biogeography and phylogeny of the genus *Symbiodinium* using the ribosomal array (Rowan and Baker, Rodriguez-Lanetty and Hoegh-Guldberg, 2003, Baker et al, 1997; Loh et al, 2001; Lajeunesse et al, 2002, 2001). From these, some ecological relevance can be inferred about the function of particular symbiont types, and predictions about the future of coral species are made based on low replication within areas. Without a thorough understanding of the level of flexibility in the coral symbiosis, we cannot accurately predict how corals will react to certain stressors and levels thereof. Here, it has been shown that each host in the family of Pocilloporid corals has multiple options in their symbiotic partnership and that these are not only optimized to the environment that the colony is growing in but is also flexible if the environment is changed. This suggests that corals may have the potential to optimize their performance to a wider environmental range than previously thought.

Even though the ITS regions have attracted some controversy as to whether they can be used as an ecologically relevant marker due to the high rates of change, the results of this study seem to validate the use of this region at this fine scale level and suggest that small differences in the ITS-2 region may indeed confer functionality as ITS2-types are regulated on a fine scale in relation to local environment. These conclusions must remain limited to the species studied, and other species of corals may not have the ability to associate with multiple symbionts, as each species does not necessarily follow the same strategy. Some species may have a multitude of symbionts suited to cover particular environments over the full range of their distribution, whereas other species may associate with a single symbiont that has a broad tolerance range over a particular environmental distribution. Of course there are many possible combinations and this diversity indicates the importance of studying these associations in depth so we can more accurately understand the intricacies involved in the persistence of a successful symbiosis. More importantly, it will yield information on the tolerance levels of each symbiont and the associations as a whole. Moreover, this will help us predict how corals will respond to changes in their environment and we can start to evaluate how damaged reef areas are most likely to respond. If this information can be coupled with large scale monitoring efforts and population genetics of the host, management programs can start to evaluate

which regions are most valuable in terms of sustaining reef health and providing viable recruits to damaged areas.

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Symbiodinium ITS-2 sequences: inter- or intraspecific data? Implications for the detection of ecological & biogeographic patterns

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Contemporary molecular systematic analyses generally employ multiple independent markers to evaluate whether conclusions drawn from individual sequence datasets are evolutionarily trivial or meaningful. The clade-level systematics of *Symbiodinium* has largely followed this approach, using data from two independent markers (cpDNA and mtDNA) to support phylogenies based on studies of nuclear rDNA (18S and 24S) (. However, fine-scale (sub-clade) relationships within this genus are unclear; the uniform application of multiple independent markers (to the same samples) is required for their resolution.

The nuclear ITS-2 region has been extensively applied to investigating sub-clade diversity within *Symbiodinium*, and sequence variants ("types") within this region have been suggested as representing different species. However, this conclusion, based on an analysis of 16 sequences (including 9 named taxa) in 2001, needs validation in light of the rapidly increasing number (>100) of distinct sequences that have been reported since.

Symbiodinium systematics and ecology: Limitations of the current framework

When interspecific algorithms are applied to *Symbiodinium* ITS-2 sequence data, the resulting phylogenies contain many unresolved polytomies and lack bootstrap support at many nodes (LaJeunesse 2002, LaJeunesse 2005). In addition, "living ancestors" (*C1* and *C3*) within clade *C* phylogenies violate assumptions of the Phylogenetic Species Concept and the algorithms used to build bifurcating trees (Figure 1 in LaJeunesse [2005]). Species distinctions within *Symbiodinium* based on ITS-2 types are in some cases made on the existence of paralogs in the rDNA repeat that differ by only a single base pair.

We suggest that systematic decisions based on these methods and criteria likely obscure important patterns in the distribution of *Symbiodinium*. LaJeunesse (2005) argues that *Symbiodinium* ITS-2 types occupy unique ecological niches (sometimes based on symbionts' host distribution, endemicity and/or rarity) and may therefore be considered distinct "species". However, explicit descriptions of these unique niches are rarely reported for types, and seldom tested statistically. The community-level sampling strategy commonly employed for *Symbiodinium* ITS-2 (in which a few samples from each of many host species are identified) has been useful in exploring the limits of *Symbiodinium* diversity but lacks the statistical power necessary to test how diverse the symbionts of particular coral species really are. This is critical for investigations of the Adaptive Bleaching Hypothesis.

Population-level theory explains some Symbiodinium ITS-2 variation

Observed patterns of *Symbiodinium* ITS-2 variation are more consistent with interpretations based on population theory than with those based on interspecific phylogenies. In populations, many ancestral haplotypes are expected to exist alongside their descendants (Templeton et al. 1992). These ancestral haplotypes are relatively common and widely distributed (*e.g.*, *C1* and *C3*), while descendant haplotypes are relatively rare and restricted in distribution (*e.g.*, paralog types). The older (and by extension, the more abundant) an ancestral haplotype, the more descendant haplotypes are predicted to be associated with it (Watterson and Guess 1977). Therefore, rare haplotypes (*e.g.*, *C1a*) are more likely to be related to ancestral haplotypes (ex: *C1*) than to other paralogs (*e.g.*, *C1b*) (Excoffier and Langaney 1989). Single base pair differences between sequence pairs detected using species-level markers are treated as intraspecific diversity (Avise et al. 1987, Templeton et al. 1992) in both soft corals and dinoflagellates (Adachi et al. 1997, McFadden and Hutchinson 2004, Shao et al. 2004). Intraspecific datasets are expected to produce multifurcating trees (Templeton et al. 1992).

Traditional clade *C* phylogenies are reminiscent of Avise's "wall of death" (Avise 2004), predicted to occur when a species-level algorithm is applied to intraspecific data. These algorithms can incorrectly depict extant ancestral haplotypes as "living ancestors", occupying a branch of length zero at the basal node of a cluster (Posada and Crandall 2001). In published bifurcating trees of *Symbiodinium* clade *C*, this is the position occupied by sequences *C1* and *C3*. Viewing *C1* and *C3* as the ancestral haplotypes of two respective species explains the existence of their many paralogs (*C1a-k* and *C3a-m*) and the lack of hierarchical organization within each lineage. LaJeunesse (2005) correctly identifies the *C1* and *C3* sequences as ancestral, but fails to arrive at the most parsimonious conclusion– that they are haplotypes, not species.

An alternative approach to Symbiodinium systematic

We suggest that much of the variation in *Symbiodinium* ITS-2 in fact represents different populations diverging from extant (and often common) ancestral haplotypes. This approach generally agrees with relationships assigned using interspecific tree-building algorithms, but recognizes clusters of closely related sequences that represent fewer, statistically better-supported "species". We propose that significant fine-scale diversity arises through mutation during the asexual reproduction of *Symbiodinium* living within hosts and is culled by selective purging events (such as bleaching). Novel symbiont haplotypes arising in this manner are not necessarily different species, even though they may achieve local abundance or patchy geographic distributions (through host fragmentation and/or vertical transmission). Our more conservative view of *Symbiodinium* taxonomy has important implications for understanding which molecular patterns are ecologically meaningful, and impacts our understanding of flexibility and specificity in coral-algal symbioses.

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Symbiont shuffling represents a trade-off for modern reef-builders

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The recent discovery of the genetically diverse nature of the dinoflagellate genus *Symbiodinium* (zooxanthellae) that forms symbiotic associations with stony corals raises the possibility that physiological properties and tolerances of reef corals may vary according to the association established. The genus *Symbiodinium* consists of at least eight clades (A to H) based on sequence analysis of nuclear ribosomal DNA (reviewed in Baker 2003, Pochon et al. 2004), as well as many genetic types within each clade referred to as subclades or strains (e.g., C1, C2). In most broadcast spawning corals, zooxanthellae are acquired from the environment in early ontogeny and become established in the endodermal cells of coral hosts as an endosymbiosis. This creates an opportunity for the host to establish an association with a variety of symbionts. Indeed, adults of some coral species form associations with more than one *Symbiodinium* strain according to the local environment or microhabitats within a coral (Rowan and Knowlton 1995, Rodriguez-Lanetty et al. 2001, van Oppen et al. 2001, Ulstrup and van Oppen 2003). Such polymorphic symbioses suggest that corals within a species may not be physiologically uniform and that the taxonomic identity of the *Symbiodinium* partner(s) may be as significant as that of the host in determining the physiology of the holobiont (host-symbiont partnership).

Initial symbiont up-take is non-selective

The apparent specificity for strain C1 observed in adult populations of *Acropora tenuis* is not present in the early stages of infection. Instead, new recruits take up a mix of C and D strains and become dominated by *Symbiodinium* clade D after ~4 months (Little et al. 2004). The lack of specificity in initial uptake of zooxanthellae in early ontogeny provides a mechanism for establishing associations with multiple symbionts and, hence, may be adaptive, as different zooxanthella types can have different physiological characteristics. The increase in *Symbiodinium* clade D is unlikely to reflect greater mortality of C-juveniles, as accumulated mortality was only slightly greater in C-juveniles three months after settlement but did not differ between C- and D-juveniles seven months after settlement. The increase in juveniles harbouring *Symbiodinium* clade D may therefore be caused either by competition between algal types or by a host-mediated up-regulation of *Symbiodinium* clade D.

Role of symbiont type in holobiont physiology

Both *A. tenuis* as well as *Acropora millepora* juveniles grow 2-3 faster when associating with *Symbiodinium* clade C compared to associations with clade D (Little et al. 2004). Faster growth of holobionts infected with *Symbiodinium* C may reflect a greater contribution of the symbiont to host nutrition through faster rates of population growth inside the host (Fitt 1985). For *A. tenuis*, the faster growth rates of C1 juveniles may explain why C1 adults are the most common at Magnetic Island (Ulstrup and van Oppen 2003). In contrast, the dominance of *Symbiodinium* D,

known to be associated with greater thermal tolerance (Baker et al. 2004, Fabricius et al. 2004, Rowan 2004) in naturally infected, 6-month-old *A. tenuis*, is more difficult to explain.

Conclusions and future directions

Shuffling from *Symbiodinium* clade C to D may increase thermal tolerance, but is likely to result in impaired growth, competition and reproduction (trade-off). Furthermore, it is currently not known how wide-spread symbiont shuffling is as a mechanism to cope with environmental change, and symbiont changes are not heritable in many species. Future work will therefore need to investigate the potential for adaptation through selection on genetic variation and new mutations in both the coral host as well as the algal endosymbionts.

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"Checks and Balances" in the identification of *Symbiodinium* diversity

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The identification of genetic diversity in dinoflagellates belonging to the genus *Symbiodinium* has been the subject of numerous studies over the last decade. During this time, a variety of molecules have been utilized. Some of these loci offer a "coarse" (i.e., 18S-rDNA) view while others provide a much "finer" (i.e., DNA fingerprinting) portrait of diversity within *Symbiodinium*. The use of sequences from the ribosomal internal transcribed spacer (ITS) region has become a standard in categorizing genetic diversity of these dinoflagellates and has revealed a plethora of ecological "types" within the genus. For some of these "types", data from microsatellite loci has uncovered additional, biologically relevant, diversity. However, one cannot help to ask if this diversity is truly "real" or results from artifacts created by molecular techniques. Here, I discuss ways in which *Symbiodinium* ITS and microsatellite data can be "checked" for methodological and interpretation errors. These processes not only lead to a “balance” in our quantification of *Symbiodinium* diversity, but also provide novel insight into the biology of these unique organisms.

The ribosomal internal transcribed spacers (ITS)

Among eukaryotic organisms, the ribosomal DNA (rDNA) operon is organized in a similar fashion. The operon consists of three ribosomal RNAs (5.8S, 18S and 28S), each separated by external and internal transcribed spacer regions. The 5.8S rRNA is separated from the small subunit (SSU or 18S) rRNA by the first of two internal transcribed spacers (ITS1), while the large subunit (LSU or 28S) rRNA and 5.8S are separated by the second internal transcribed spacer (ITS2). Typically, these three rRNAs, as well as both the internal and external spacers, are transcribed by RNA polymerase I into a single precursor molecule, the 35-45S pre-rRNA. This molecule undergoes a series of processing steps that ultimately leads to mature and fully functional rRNAs. Mutations in the spacer regions flanking the rRNAs are known to prevent formation of the mature molecules, suggesting that they contain essential signals required for correct processing. In recent years, both ITS1 and ITS2 have been recognized as vital components of the processing steps leading to rRNA maturation; specifically, it has been emphasized that a particular secondary structure in these regions are required for correct processing (Coleman 2003). This being the case, novel *Symbiodinium* ITS sequences could potentially be validated by comparing them to established secondary structures for the genus as well as specific *Symbiodinium* clades. For this reason, the ITS2 secondary structures from members of the eight major *Symbiodinium* clades, A-H, were elucidated and compared.

The ITS2 secondary structure of Symbiodinium

In spite of large amounts of primary sequence divergence (>60%, in some cases), a nearly common ITS2 secondary structure has been recovered from representatives of all *Symbiodinium* clades (Fig. 1). This structure is consistent with the four-helix model, which has been previously described from other organisms (Coleman 2003), including some free-living dinoflagellates (Gottschling and Plotner 2004). However, among *Symbiodinium* clades B, C, F and H, a subtle, but significant, difference in

secondary structure was apparent when compared to the other *Symbiodinium* clades, free-living dinoflagellates and eukaryotes in general. This structural difference was the presence of an additional stem-loop (labelled IIIa in Fig. 1), which results in a five-helix model for these clades. By using the features inherent to this ITS2 secondary structure, such as nucleotide bulges and conserved processing sites, as well as compensatory base changes found among groups of closely-related taxa, the structural skeleton for *Symbiodinium* ITS2 provided here will find use in the validation of novel sequences and optimisation of alignments for phylogenetic reconstruction.

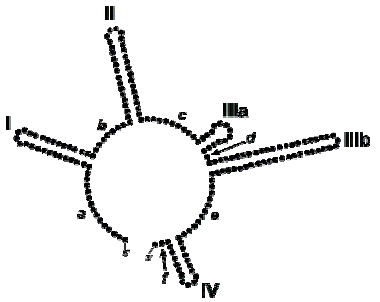

Fig. 1. Consensus *Symbiodinium* ITS2 secondary structure

Microsatellites

Microsatellites are simple, tandemly repeated DNA sequences elements, distributed abundantly in the genomes of virtually all organisms. For *Symbiodinium*, data from microsatellite loci (*e.g*., either the presence or absence of an allele, size variability between alleles or phylogenetically informative substitutions in the flanking regions adjacent to the repeat array) have demonstrated the non-representative nature of some zooxanthella cultures. When compared to the populations from which they were established (Santos et al. 2001), this data confirmed that *Symbiodinium* spp. are haploid in the vegetative life stage (Santos and Coffroth 2003), revealed striking differentiation in *Symbiodinium* populations associated with the octocoral *Pseudopterogorgia elisabethae* across the Bahamas (Santos et al. 2003) and elucidated fine-scale diversity and specificity in the most prevalent lineage of symbiotic dinoflagellates of the Caribbean, *Symbiodinium* "type" B1 (Santos et al. 2004). However, to date, the mutational behaviour of microsatellite loci in the *Symbiodinium* genome has not been discussed. Data from two well-characterized *Symbiodinium* microsatellites suggest that a range of evolutionary processes operate on these loci.

Evolutionary patterns in Symbiodinium microsatellites

Substitutions, nucleotide insertion and deletions (indels), alterations to the repeat array structure and non-stepwise changes in repeat number have been documented from these two *Symbiodinium* microsatellites. Because the accurate estimation of population structure and relationships using microsatellite data rests in the assumption that alleles identical in state (i.e., size) have experienced a common mutational history, this discovery may complicate future studies. However, although microsatellite alleles homologous in size but resulting from different evolutionary processes (e.g., size homoplasy) were identified, this phenomenon appears to pose little problem for the interpretation of population structure if techniques capable of detecting differences in the primary sequence of microsatellite alleles are employed. Furthermore, mutations such as the ones described above are a rich source of information that complement and extend the population-level data inherent to these markers. Analysing such mutational patterns will identify the forces shaping the genome of these important organisms and provide new insight into *Symbiodinium* biology.

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Transcriptome analysis of a cnidarian – dinoflagellate mutualism reveals complex modulation of host gene expression

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Mutualistic symbioses are defined as the association between unrelated organisms living together in a close, protracted relationship that benefits both partners. Cnidarian – dinoflagellate associations represent one of the most important symbioses in the marine environment. These partnerships form the trophic and structural foundation of coral reef ecosystems, and have been the driving force in the radiation and biodiversity of cnidarian species. Despite the prevalence of these marine symbioses and the overall interest in coral reef health, we still know very little about the cellular and molecular basis of the intracellular cnidarian – dinoflagellate symbiosis. What are the key molecular modulators that initiate, regulate and maintain the interaction between these two different biological entities?

Modulation of host gene expression as a function of symbiosis

The discovery and identification of host genes that modulate cnidarian – dinoflagellate symbioses is a topic that is ideally suited to a comprehensive microarray approach. We conducted a comparative host anemone transcriptome analysis using a cDNA microarray platform to identify genes involved in cnidarian – algal symbiosis. Following earlier proteomic studies [1], we used the temperate anemone *Anthopleura elegantissima* as a model as it occurs naturally in both the symbiotic and aposymbiotic state (Figure 1). We discovered 91 unigenes (5.63% of the genes originally spotted on the microarray) that showed to be significantly different between aposymbiotic and symbiotic anemones. From the identified host unigenes only 32 genes showed significant BLAST hits (E < 1.0 x 10-4) with homologue known genes in the Genbank.

Fig.1 Symbiotic (brown) and aposymbiotic (white) anemones of *Anthopleura elegantissima*

One important discovery is that our data do not support the existence of symbiosis-specific genes involved in controlling and regulating the symbiosis. Twelve of the 91 differential unigenes were very highly expressed in symbiotic state (Fig. 3). These were viewed with suspicion as possible algal genes that were contaminating the host-only cDNA library. Specific primers

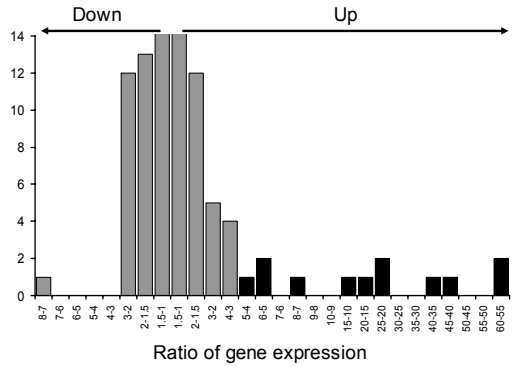

Fig. 2. Distribution of genes as a function of the ratio of expression. Arrows divide those down and up regulated in symbiosis. Color bars mean as follow: Black (contaminating algal symbiont unigenes) and Grey (host unigenes).

for these highly symbiotic unigenes were constructed and used in PCR reactions with genomic host-only and algae-only symbiont template DNA. Successful DNA amplification was only achieved in the algal DNA samples and not in the host genomic DNA (data not shown). These contaminating algal unigenes were therefore removed from further analyses. All other up-regulated host unigenes in the symbiotic state were also expressed in some extent in aposymbiotic hosts, as their fold change expression was subtle; largely ranging between 1 and 2. Rather than finding genes which expression change as a function of symbiosis in an ON/OFF manner, we detected alterations of expression of genes regulating different functional processes (Fig. 3).

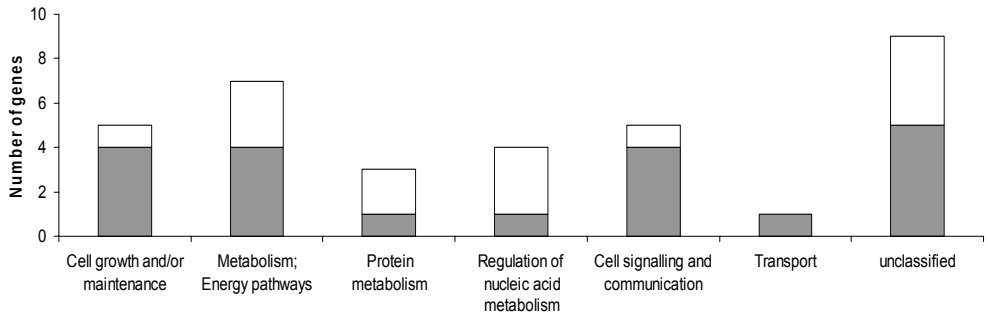

Fig. 3. Distribution of the known host unigenes based on their functional classification. White and grey bars are the genes up and down regulated in symbiosis, respectively.

Gene ontologies of the 32 differentially expressed genes exhibiting significant homologies reveal the complexity of the interaction between the symbiotic state and host gene expression. This suggests that symbiosis is regulated and controlled, not by pathways unique to the symbiotic state but rather by changes within existing pathways used to control metabolism and growth of the animal as whole. Most of these genes are putatively involved in metabolism and energy pathways, cell growth and/or maintenance, and cell signaling.

Suppression of apoptosis and deregulation of cell cycle

We discovered that the gene expression of key biomolecules involved in cell cycle progression and apoptosis are differentially modulated in symbiosis. For instance, we detected a down regulation of the gene Sphingosine Phosphate Phosphatase (SPPase) in symbiotic state, which may play a role in keeping the levels of the anti-apoptotic sphingolipid, Shingosine-1-Phosphate (S1P) higher over the pro-apoptotic sphingolipid, sphingosine. This increase of S1P would facilitate higher survival of symbiont-harboring host cells, as it has been documented to occur in other animal cells [2]. Moreover, high levels of S1P would also enhance cell proliferation by expediting the G1/S transition in the cell cycle [3].

Our findings provide novel insight into the physiological roles of sphingolipids in cnidarian – algae symbiosis, and how the modulation of sphingolipid regulators, such as SPPase, is emerging as putative mechanisms to regulate host cell apoptosis and survival in host – symbiont associations. We suggest that a suppression of apoptosis together with a deregulation of the host cell cycle create a platform that might be necessary to symbiont survival and/or symbiont-containing host cell survival. These findings are very interesting as they adjust our perception of the interaction between cnidarians and symbiotic dinoflagellates from a cellular perspective. We have always

visualized the cnidarian – algae interaction as a cooperative since the ecological outcome of the interaction is a mutualistic symbiosis; however, from a cellular level the interaction between host and symbionts appear to have components of a parasite/pathogenic interaction. Symbionts, like pathogens need to overcome the host innate immunity to enter, reside and growth inside the host cell. But intriguingly, the algae-induced changes in the host cell, which show some similarity to pathogen – host interactions, do not lead to the development of disease. Understanding the nature of the molecular regulation of cnidarian – algal symbiosis and by comparison with parasite/pathogen associations, it will provide further insight into the evolution of host – symbiont/parasite associations.

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Consensus statement on the current understanding of the diversity, specificity and flexibility of *Symbiodinium* symbioses.

Statement agreed to by participants at Puerto Morelos meeting (May 17 2005)

Dinoflagellates in the genus *Symbiodinium* are the principal symbionts of reef-building corals as well as hosts from several other phyla. These single-celled photosynthetic organisms generally occur intracellularly within host cells. Once thought to represent a single species, *Symbiodinium microadriaticum*, they are now considered to be phylogenetically diverse and include a number of described and undescribed species.

Understanding the forces that have driven the distribution and evolution of *Symbiodinium* may provide important insights into the response of corals to environmental change. Distinguishing which processes operate at ecological as opposed to evolutionary timescales is critical to this endeavour. Future efforts should focus on relating genetic diversity to ecologically relevant physiological differences.

Corals and their dinoflagellate symbionts exhibit a range of specificities. Some coral species transfer symbionts directly between generations, while others acquire symbionts from the environment anew each generation. In the latter case, the events that lead to the establishment of *Symbiodinium* symbioses are relatively specific despite the fact that most genotypes can be taken up by host cells initially.

The initial types of *Symbiodinium* that enter newly settled corals appear to be a subset of those available in the environment. This set of *Symbiodinium* types is further narrowed down to the complement dominant within the adult host and its environment, although some types may persist at background levels within the tissues of the host coral.

The processes by which one or several symbionts become dominant within the host have yet to be described, but probably involve host-symbiont recognition, specific host factors and competition between *Symbiodinium* genotypes. Future studies need to focus on understanding these mechanisms and their relative importance.

Adult corals may change their symbiotic complement in response to environmental change. ‘Shuffling’ and ‘switching’ are two non-exclusive mechanisms by which this may be accomplished. ‘Shuffling’ is a quantitative (compositional) change in the relative abundance of symbionts within a colony; ‘switching’ is qualitative change involving symbionts acquired from the environment. These exogenous symbionts may represent types that are new to the colony but not the species, or may be truly novel to the host species which is referred to as ‘evolutionary switching.’

While evolutionary switching is assumed to explain the phylogenetic patterns of symbiont distribution within hosts, such events are thought to be very rare. Shuffling and/or switching of existing symbionts are thought to be more common. However, distinguishing between evolutionary (and truly novel) switches, and those that involve existing symbionts, is a methodological challenge.

Some corals routinely shuffle symbionts as a consequence of seasonal regulation of symbiont numbers with or without visual signs of bleaching. Corals can also shuffle symbionts during and after bleaching. Switching is also likely to be promoted by seasonal regulation and bleaching. Both shuffling and switching may be important

mechanisms that extend the ability of corals to acclimatize to changes in the environment but requires further investigation to demonstrate true physiological advantages of the changes involved.

Bleaching probably did not evolve directly as a mechanism for shuffling or switching symbionts, and has clear pathological effects. A potential side-effect of bleaching is an acceleration of symbiont change which has the potential to elevate the tolerance threshold of coral reefs to environmental change. However, without evolutionary switching, there will be no change in the tolerance threshold for any particular coral-*Symbiodinium* symbiosis.

There continues to be widespread concern within the research community over the future of coral reef ecosystems. Increased sea temperatures as well as ocean acidification and other anthropogenic challenges continue to pose grave threats to the future of these diverse ecosystems and the people that depend on them. Unless these threats are addressed as a priority soon, coral reefs will continue to degrade.

Scott Santos addresses the workshop on “Diversity, flexibility, stability, physiology of Symbiodinium and the associated ecological ramifications”.

Theme 3: Exploration of the Coral and Symbiodinium genomes (May 19-21, 2005)

Co Chairs: Bill Leggat, Sophie Dove, David Yellowlees

Over the past five years, there has been a rapid growth of studies aimed using molecular methods to explore the gene expression of corals and *Symbiodinium* using molecular methods. This discussion evolved out of the perceived need to promote better communication and synthesis between research groups in this area. The major goal of this section of the workshop was to review the key research questions and agenda for the future and where possible develop synergies and collaborations.

Participants

Andrew Baker; Merideth Meredith Bailey; Jeffry Deckenback; Sophie Dove; Susana Enriquez; William Fitt; Ruth Gates; Ove Hoegh-Guldberg; Roberto Iglesias Prieto; Michael Lesser; Todd LaJuenesseLaJeunesse; Mikail Matz; David Miller; Mckenzie Manning; Maurico Rodiguez-Lanetty; Adrienne Romanski; Hector Reyes; Baraka Ruguru.; Eugenia Sampayo; Jodie Swartz; Roee Segal; Noa Shenkar; Madeleine van Oppen; Shakil Visram; Gidon Winters; Mark Warner; David Yellowlees and Assaf Zenvoluni.

Theme coordinator Bill Leggat takes notes during discussions. Others in photo – Mark Warner (left) and Ross Hill (right).

Progress in coral genomics

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Anthozoan cnidarians such as corals are phylogenetically basal, and a substantial body of morphological and molecular data supports the idea that they are the closest extant relatives of Ur-eumetazoa – the common ancestor of all higher animals. To better define the basic metazoan gene complement, and also gain insights into the genetic bases of coral-specific properties such as calcification, we are conducting ESTs analyses on two anthozoans – the coral *Acropora millepora* and the sea anemone *Nematostella vectensis*. To date, we have examined 16,571 non-redundant ESTs (12,547 predicted peptides) across the two species, and both projects are ongoing. The resulting dataset is much more complex than might be assumed based on morphology, and implies that much of the genetic complexity normally assumed to have arisen much later in animal evolution is actually ancestral. For example, all of the key developmentally-regulated cell-signaling pathways are represented, in most of the types usually associated with vertebrate development. At least 5% of genes have been independently duplicated in the anthozoan lineage, and emerging data for other cnidarians suggest complex patterns of duplication in some cases. The most surprising implication of our analyses, however, is that anthozoans have retained a substantial number of genes not previously known in the animal kingdom. These and other anomalously distributed genes suggest unanticipated physiological properties, and therefore that anthozoan stress responses may be complex. The redundant Acropora EST collection, comprising almost 13,000 ESTs from three developmental stages, has been printed to microarrays and we are using these to investigate many aspects of development as well as stress responses. The arrays are available from the Adelaide microarray facility at $US160 (or $A200). A third generation Acropora microarray is in development – this will add at least 4,000 ESTs from zooxanthellae-free adult coral tissue to the previous release.

A microarray approach to understanding stress responses and the functional biology of corals

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Mass coral bleaching events have greatly increased in frequency and intensity over the past 30 years, leading to widespread concern over the long-term survival of coral reefs as we know them today. Anthropogenic toxicants from runoff and ship groundings have also been reported to cause localised bleaching (Jones et al. 2003; Smith et al. 2003). Coral bleaching has been studied at the level of the photosystems of symbiotic algae (e.g. Warner et al. 1996, 1999; Jones et al. 1998, 2001; 2003; Hill et al. 2004; Tchernov et al. 2004), however, the molecular responses of the coral partner in relation to heat or toxicant stress are almost completely unknown. The limited molecular studies conducted to date on stress responses in corals have focussed on single candidate molecules, such as Hsp70, often using heterologous probes or antibodies to assay changes in protein levels. For example, Brown et al. (2002) examined a range of host and symbiont biomarkers in heat-stressed *Goniastrea aspera* colonies and showed that the coral host is likely to play a significant role in enhancing the thermal tolerance of certain tissues by increased production of CuZnSOD, Hsp60 and Hsp70 at elevated temperature.

The availability of a microarray chip developed for the coral *Acropora millepora* by James Cook University and the ARC Centre for the Molecular Genetics of Development, as well as the availability of field coral samples from two non-bleaching (’00-’01 and ’02-’03) and one bleaching (’01-’02) summers collected by the Australian Institute of Marine Science, permits us to study the natural response of coral to thermal stress using microarray technologies. In addition, we have exposed *A. millepora* larvae to elevated temperatures and various concentrations of the heavy metal copper, the antifoulant TBT and the herbicide diuron in replicated exposures for 24 hours, allowing us to examine gene expression differences between these stress treatments. . As a wide range of stressors are known to cause corals to bleach, the specific cellular mechanism underlying the bleaching response is likely to be different for each stressor. The approach proposed here aims at characterising these differences and may lead to the development of diagnostic tools for use in the field.

Materials & methods

The coral *Acropora millepora* was used for this study as this species is common on the Great Barrier Reef and easily identifiable, it is relatively well-studied and coral husbandry methods have been developed for this species. A pilot microarray experiment was conducted, where RNA was isolated from 6 tagged coral colonies sampled on 24 January 2000 (a non-bleaching summer) and the same 6 colonies on 24 January 2002 (a bleaching summer). Gene expression levels at ~3,000 ESTs were compared between the two sampling time points.

Results and Discussion

Over a hundred genes consistently showed significant differential expression in the bleaching and non-bleaching comparison. However, of the differentially expressed genes, none showed a fold change higher than three. One of the most highly down-regulated genes is clearly related to a human gene that has been implicated in cancer metastasis, suggested the possibility of involvement of the coral gene in expulsion of zooxanthellae. We are currently validating these results using real time quantitative PCR. One of the challenges we are facing is the identification of appropriate house-keeping genes for comparative analysis.

Conclusions and future directions

Real time PCR will aid in selection of the most informative time points during a natural bleaching event to use in a future microarray experiment. We will also be targeting some genes directly using real time PCR, such as some of the genes encoding heat shock proteins and homologs of the bacterial universal stress proteins.

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Coral Reef Genomics: A Genome-wide Approach to the Study of Coral Symbiosis

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The symbiotic association between corals and *Symbiodinium* is one of the structuring features of coral reef ecosystems. The dramatic decline in the health of coral reefs worldwide has prompted great interest in understanding how the symbiosis breaks down under conditions of environmental stress. Equally important is an understanding of mechanisms that lead to the establishment and regulation of the "normal" state of symbiosis. We are taking a genomics approach to identify genes and gene networks in both partners that play a role in the establishment of the symbiosis. To accomplish this, we are developing cDNA libraries and cDNA microarrays to study the onset of symbiosis in two Caribbean corals, *Montastraea faveolata* and *Acropora palmata* and several strains of *Symbiodinium*. These corals spawn egg/sperm bundles that lack zooxanthellae, providing an experimental system for studying the onset of symbiosis. We rear the larvae and then experimentally establish the symbiosis, using cultures of *Symbiodinium*. We sample RNA from both partners throughout the process to capture genes that are expressed at different stages of symbiosis. We have created cDNA libraries from coral eggs, embryos, larvae, and adults, and from coral larvae infected with *Symbiodinium*. We are currently making cDNA libraries from the *Symbiodinium* strains with which the larvae were infected. To date, we have generated over 10,000 ESTs and are developing methods to annotate the ESTs to obtain as much information from the sequence data as possible. Using the sequence information, we will select specific genes to include in microarrays. We have conducted a pilot study using a 100 gene cDNA microarray to examine relative gene expression levels in coral eggs vs. adult tissues. We are now scaling up to examine gene expression levels in thousands of genes in various stages of coral development, as well as in symbiotic vs. non-symbiotic larvae. To examine the genomic context of genes that we identify as being related to symbiosis, we have made BAC libraries from both coral species that can be probed and examined for upstream regulatory sequence information.

Targeted Functional Genomics of Coral Stress

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The recently documented trend of gradual decline of coral reefs on a global scale is a matter of high concern. Our awareness of the problems experienced by corals is critically dependent on the ability to recognize stressed condition, as well as determine the cause and magnitude of stress. The symptom of stress most frequently quantified during field surveys is “bleaching” - loss of pigmentation due to dramatically reduced number of algal endosymbionts (“zooxanthellae”) within coral’s tissues (Jokiel and Coles, 1990). The bleached corals often die, sometimes on the scale of the whole reef, although in many cases they are able to recover (McClanahan, 2004). Although easy to observe in the field, the “bleaching” symptom lacks the in discriminatory power: it signifies only the most severe stress and can be caused by a variety of factors such as increased (Jokiel and Coles, 1990) or decreased (Saxby, et alDennison and Hoegh-Guldberg, 2003) water temperature, elevated visible light (Lesser and Farrell, 2004), low salinity (Kerswell and Jones, 2003), chemical insult (Brown, 2000), and bacterial infection (Ben-Haim et al., 1999). Most importantly, without additional information it is not possible to predict whether the bleached coral is going to die or recover.

Clearly, methods integrating several observable parameters that may change during stress have a better chance to infer the details of the coral condition. We believe that monitoring expression levels of 100-150 genes that respond to stress will provide sufficient data to evaluate stress intensity as well as recognize the contribution of particular stress factors. The first goal of our project is to develop such a technique. We performed six subtractive hybridizations to obtain cDNA samples enriched by the transcripts that are either up- or down-regulated in *Porites lobata* and/or *Porites compressa* during any of following the three types of stress: elevated heat/light, exposure to copper and mechanical injury. The array of 3456 randomly picked clones from these samples has been printed. Its preliminary characterization indicates that the subtractive cDNA libraries contain a high proportion of differentially expressed genes: 285 clones have been identified thus far as possible candidates, some of them common, some specific to particular stressors (Fig. 1). This array will be further extended by adding similar subtractive analysis of three more stressors – cold, excessive light (but no temperature increase) and low salinity. Our aim is to discover at least 150 stress-related genes, expression of which will be it quantified in corals stressed under a variety controlled laboratory conditions. The results will be subjected to multivariate statistical analysis to extract patterns of gene up- and down-regulation characteristic for different stressors and stress intensities, using the same algorithm that was recently developed for artificial olfaction (Carmel et al., 2003).

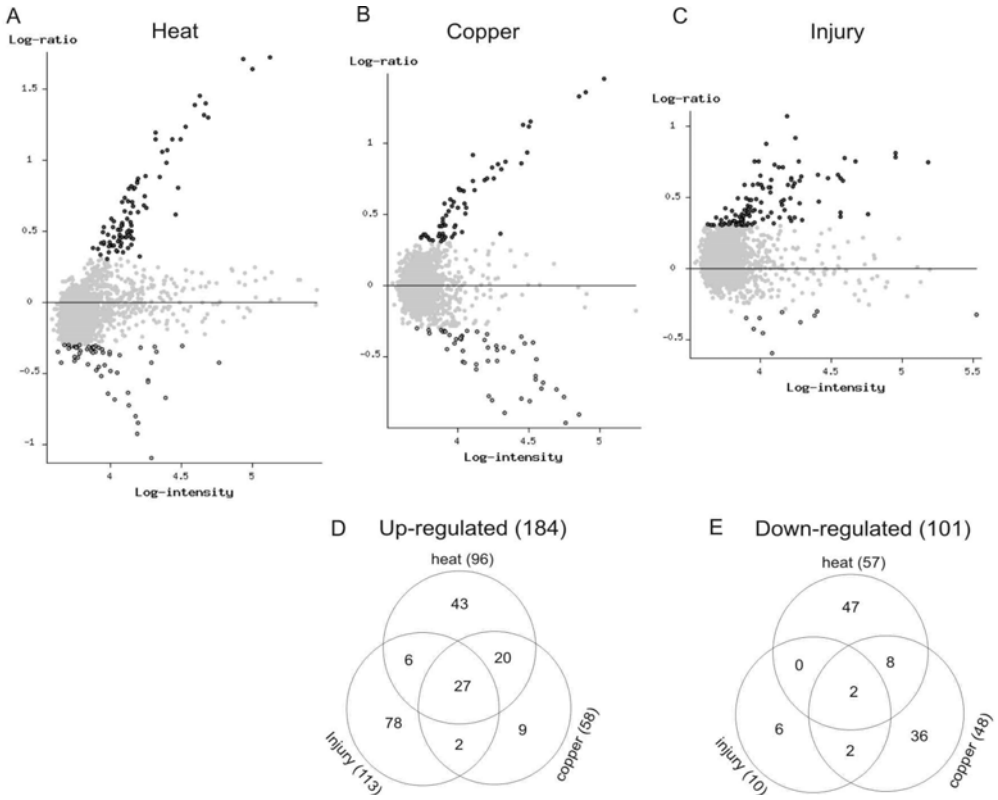

Fig. 1. Results of the primary array analysis of coral stress. A-C: Scatter plots of the intensity ratio between two compared samples at each spot versus mean spot intensity, in logarithmic coordinates. Spots significantly deviating from the mean are highlighted: the ones above zero are up-regulated, the ones below - down-regulated. D and E: numbers of common and unique differential spots for the three types of treatment, following the results represented on panels A-C. The plots and lists of differential spots were generated using the NIA Array Analysis tool

The identification of the genes up- and down-regulated during stress will provide an opportunity to investigate the molecular mechanism of stress response in corals, which is the second goal of our project. We are going to sequence all the clones showing expression changes in our experiments, obtain full-length cDNA sequences for them to facilitate the analysis of their homology relationships, and compare the resulting collection of genes to the known stress-related gene interaction networks from other organisms. The information about the correlation between expression levels of individual genes in our experiments, with reference to the organization of characterized gene networks, will provide additional grounds for inference of the stress-related gene regulation network in corals (Herrgard, et al Covert and Palsson, 2003). It can be expected that the newly identified network elements may serve as key indicators of survival ability of corals under stress. For example, the expression level of genes responsible for the non-specific stress response may reflect the general susceptibility of the organism to stress. In the future, such knowledge will provide the basis for assessing survival chance of different coral species in different ecological zones and/or geographical regions in the face of changing global conditions.

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Using Molecular Markers – A Cautionary Tale

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The SSU (18S), ITS-1, ITS-2 and LSU (28S) rDNA are arguably the most common markers used for exploring the taxonomy of dinoflagellate symbionts belonging to the genus *Symbiodinium* (reviewed by Baker, 2003; Coffroth and Santos, 2005). These four regions of the ribosomal array provide different levels of taxonomic resolution based on their individual rates of evolution and thus provide insight into a variety of fundamental aspects of coral biology that cross multiple temporal and spatial scales. Collectively work exploiting these markers has dramatically improved our understanding of the relationship of *Symbiodinium* with respect to other protist phyla, revealed differences in host/symbiont specificity and niche preference in corals across environmental gradients, allowed for the description of geographic patterns of distribution, and resolved aspects of the temporal and spatial stability of given symbionts with respect to their hosts and/or a particular geographic province.

An accurate and repeatable methodology is key to describing the diversity of symbionts found in cnidarians and is an endeavour that is currently a component of many studies that are being conducted on corals. The ITS-2 provides “species” level resolution within the genus (Lajeunesse, 2001) and thus was an ideal choice for the work ongoing in my own laboratory. To implement the day-to-day use of this marker in defining symbiont diversity, we conducted a series of preliminary studies and validation steps with this marker, and in doing so, encountered a number of problems that we feel have relevance to the coral reef community.

The first issue unfolded as a consequence of our inexperience using the screening methodology that has been developed for ITS-2, namely denaturing gradient gel electrophoresis (DGGE). This technique allows for rapid screening of multiple samples and promotes the identification of new sequences types based on new band positions on a gel, which can then be validated using sequencing. In our preliminary runs using this technique to explore symbiont diversity in ITS-2 amplified from genomic DNAs isolated from *Porites lobata* and *Porites evermanni*, we obtained complex banding patterns that were difficult to interpret. For speed, simplicity and familiarity with the technique we chose to clone the PCR products as a means of separating the individual ITS-2 types in the mixture for sequencing rather than using DGGE. It is noteworthy that these two species had previously been analyzed from the same geographic location and designated as containing ITS-2 type C15 symbionts using DGGE (LaJeunesse et al., 2004).

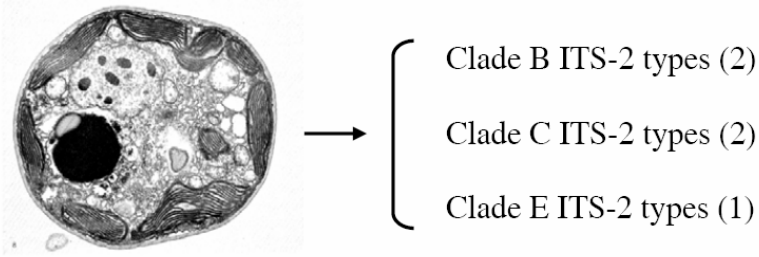
A survey of multiple clones originating from 6 colonies of *P. lobata* and 3 colonies of *P. evermanni* revealed that they contained from 1 to 6 and 3 to 8 ITS-2 types respectively, all of which belonged to clade C. A number of the newly characterized sequences were shared by both species and multiple colonies within a species, and the most commonly encountered ITS-2 type was a previously undescribed ITS-2 sequence type found in all six colonies of *P. lobata* and two of the three *P. evermanni* colonies. Other novel sequences were only found in a single colony of one species. Interestingly, C15 was only identified in only 4 of the 8 clone libraries and was not the dominant type present in either coral species. Cloning has been criticized as a downstream analytical tool in assessing symbiont diversity because of the potential

for introducing PCR and cloning error and generation of non-biologically relevant variants. In reality, the generation of sequences using clone libraries is achieved using fewer rounds of PCR than DGGE and cloning is prone to less error than PCR.

In an attempt to reconcile the large discrepancy between the ITS-2 diversity obtained using a cloning approach and that reported as a result of DGGE, we examined how our characterized cloned sequences behaved in DGGE. These analyses revealed that very closely related sequences (1–3 bp difference) exhibited different migration rates on the denaturing gradient gels, while sequences that exhibited higher level of variation (14 bp change) migrated to a very similar position on the gel . The position of bands on the DGGE is used as the criterion for selecting potentially new sequences for further characterization. Those that occupy well-documented positions are often bypassed and defined according to the initial sequence obtained for a band at that specific location. Our results suggest that selecting new bands for further analyses using this methodology may be skewed towards those bands that show the least degree of sequence divergence and has the potential to completely overlook novel sequences with the same migration characteristics as already defined bands.

The ITS-2 is considered to be a “species” level marker and is thus is widely utilized to examine fundamental questions in coral biology. Assigning an individual membership within a group based on ITS-2 sequence identity is grounded in the assumption that each DNA sequence obtained is representative of an individual symbiont. Although it is well known that ITS-2 is a multi-copy marker, for other systems it has been clearly demonstrated that the multiple sequence copies within a genome are subject to concerted evolution. This is a process by which differences in re-iterated copies of a sequence within a genome become homogenized and thus in molecular analyses appear as a single sequence type that is representative of the complement of copies in the cell (e.g. Hillis et al., 1991). To validate this assumption for ITS-2 in *Symbiodinium*, we developed an analytical protocol that allows us to PCR amplify the marker from individual symbiotic cells. Our preliminary data show that individual cells freshly isolated from the sea anemone *Aiptasia pulchella* possess up to seven sequence copies of the ITS-2 and that these copies are not the same (Figure 1). In fact, the sequence copies from a single symbiont cell represent ITS-2 identities belonging to three different clades, B, C and E.

Figure 1. ITS-2 types amplified from a single cell – number of types within clade in parenthesis.



We have ruled out methodological error as a possible explanation for these finding and our results have now been independently verified in a similar study exploring ITS-1 copies in single *Symbiodinium* spp. cells isolated from *Acropora millepora* (van Oppen et al., 2005). These data demonstrate that in both the ITS-1 and 2 regions of the ribosomal array, the intragenomic ITS sequence copies are not uniform or

homogeneous. As such, the underlying assumption that the isolation of one or multiple ITS sequence infers the presence of one or multiple individual, respectively, is not met. Perhaps equally complicating from an interpretational standpoint is the lack of a predictable pattern in the ITS-2 types belonging to different symbionts individuals isolated from the same host. We have found that in some cases an individual symbiont contains multiple ITS-2 types belonging to three different clades, as shown in the example above, but other individuals possess multiple ITS-2 types belonging to one only one clade.

In conclusion then, our data suggest that: 1) the diversity of ITS-2 sequence types within individual coral colonies is greater than previously reported; 2) the diversity ITS-2 sequence types varies among colonies of the same species; 3) the prominence of a single band on a DGGE gel does not always infer dominance of a single sequence type in a mixture; and 4) there is significant variation of ITS-2 sequence types within individual symbiont cells (intragenomic variation) that confounds the interpretation of the data generated using this marker. Collectively these data highlight the importance of validating the molecular methodologies used to explore biological diversity, contextualize a re-evaluation of the current literature based on ITS-2 sequence data, and lastly, provide the rationale for the development of new single copy markers for exploring diversity within the genus *Symbiodinium*.

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Distinct differences in a Symbiodinium EST library compared to other dinoflagellates

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Introduction

Coral reefs are dependent upon the symbiosis between the dinoflagellate *Symbiodinium* sp. and their coral host. Coral are now severely threatened by a variety of stressors, ranging from localised (point pollution) to broad-scale anthropogenic stresses [2] (river run off, global warming). How the coral symbiosis will respond to these stresses is dependent upon the genetic complement that the individual partners, and therefore the symbiosis as a whole, contain. For example, mass coral bleaching, where large portions of the symbiontic dinoflagellate population symbionts areis expelled from the coral host, often resulting in death of the host, have been correlated to increases in seawater temperatures only slightly above long term summer averages, presumably driven by global warming [3]. This slight increase in temperature leads to a breakdown of the photosynthetic capacity of the alga, the exact mechanism of which is not clearly understood [4-6], and subsequent expulsion of the alga from the host. In this case how the symbiosis as a whole responds to the thermal stress is dictated by the proteins which are expressed by *Symbiodinium*. Therefore a greater knowledge of the repertoire of proteins that are present in each of the symbiotic partners may provide clues to how they will respond current and future stress events.

Until recently our knowledge of the genetic complement of *Symbiodinium*, and dinoflagellates as a whole, was extremely limited. In 2002 only 32 protein sequences had been obtained for all dinoflagellates, some of which indicated that dinoflagellates were significantly different from any other photoautotroph. The presence of a form II Rubisco [7], previously found only in anaerobic purple non-sulfur bacteria, ; a chloroplast genome replaced by plasmid-like minicircles, encoding an extremely reduced number of genes [8,9], ; and a unique light harvesting protein (peridinin-chlorophyll *a* binding protein, PCP) [10] all indicated a unique genetic complement in dinoflagellates. Since then there has been an explosion in the number of sequences available for dinoflagellates, with four large scale expressed sequence tag (EST) projects [1,11-13], totalling over 10000 sequences, being released. However all of these studies used long term algal cultures grown under normal (non-stressful) conditions. With this in mind we constructed and analysed a cDNA library for stressed *Symbiodinium* (short term heat stress, long term heat stress, increased ammonia, increased inorganic carbon) with the aim to better understand the genes that dinoflagellates, in particular *Symbiodinium,* express under stressful conditions.

Analysis of the Symbiodinium EST library

A total of 2447 clones from a *Symbiodinium* cDNA library were randomly picked and sequenced from the 5'end, with an average of 654bp of sequence obtained for each clone. A total of 1698 ESTs were found more than once, and were grouped into 594 clusters/contigs giving a total of 1343 unique EST sequences. Given the large number of dinoflagellate ESTs recently deposited in the GeneBank, the

Symbiodinium sequences were blasted against these ESTs. Of the 1343 ESTs obtained for *Symbiodinium*, 102 were found to match sequences from other dinoflagellates with a bit score greater than 100. Translated blast searches against NCBI protein database identified 530 sequences that had bit scores greater than 50, indicating a match. The distribution of these EST was analysed and sorted by organism "best hit" and by function, where it could be assigned. This distribution was then compared to the distribution of genes from another dinoflagellate EST project (*Lingulodinium polyedrum*[1]) (Figure 1). The distribution of the *Symbiodinium* sequences was significantly different from that seen in *L. polyedrum*. Metabolic genes (25%) were the largest category identified, followed by proteins associated with cell communication (13%) and transcription/translation (13%) in *Symbiodinium*. In *L. polyedrum* the largest categories were metabolism (25%), transcription/translation (21%) and unknown (17%). Perhaps the most significant difference was associated with stress and defence genes where many more were identified in *Symbiodinium* (7% vs 1%), reflecting the stressful conditions used to generate this library. The organism group with the greatest number of protein matches were vertebrates (32%), the majority of which were metabolic (26%), transcription/translation (18%) or cell communication (14%) proteins. The alveolates as an entire group (including dinoflagellates) were next greatest (alveolates 11%, dinoflagellates 8%) followed by the prokaryotes and land plant (13% each). Again this was significantly different from *L. polyedrum*.

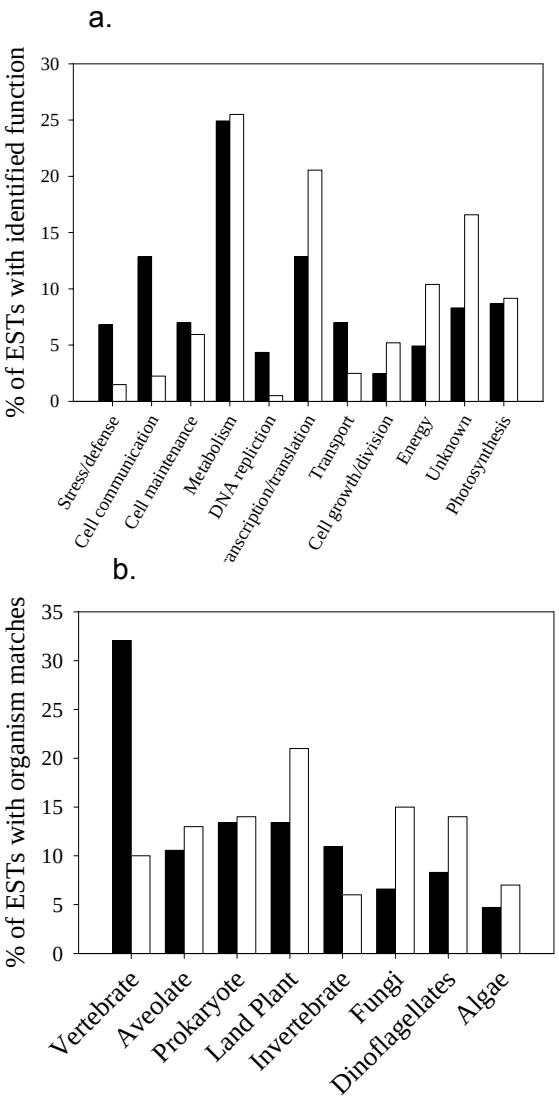

Figure 1 Distribution of proteins identified through Blastx with a score greater than 50. a. Distribution by function. b. Distribution by best hit to organisms. Black bars = *Symbiodinium* (this study), white bars from the dinoflagellate *Lingulodinium polyedrum* [1].

Conclusions

This study represents the first large scale exploration of the *Symbiodinium* genome and the first attempt to examine the stress response genes of dinoflagellates. Initial results demonstrate that the stress conditions used to generate this library has up-regulated a set of genes which have not been previously characterised from dinoflagellates. Analysis of these genes should enable use to determine how the expression of these genes respond during stress events.

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How does the Symbiodinium EST database add to our knowledge of zooxanthellae and their metabolism?

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Knowledge of the metabolism of corals and their symbionts is an essential aspect of understanding how these unique organisms function and thrive in their natural environment. A detailed appreciation of their metabolism and how it can change in response to various factors can also inform environmental management decisions. In the past metabolic studies have been addressed using standard biochemical techniques of enzyme isolation and metabolite measurement. These have been fraught with difficulties because of the small tissue volume available in corals. Progress has therefore been slow and our knowledge of carbon metabolism let alone nitrogen and phosphate assimilation is poor.

The availability of molecular techniques has the potential to revolutionise our understanding of coral metabolism and the availability of the coral EST library will be instrumental in this. We have now established an EST database for zooxanthellae which, while not as extensive as that of coral, will be of major assistance in defining some of the major aspects of metabolism, including photosynthesis, in *Symbiodinium* and defining possible differences between the different clades. The library was developed using corals (and zooxanthellae) that had been exposed to a number of stress regimes including thermal, nutrient addition (ammonium), and the depletion and addition of inorganic carbon. This was to maximise the expression of genes crucial to the management of these conditions.

Analysis of the database has identified a number of avenues for future investigation which will significantly increase our understanding of the metabolism and other processes in zooxanthellae. This includes the identification of Hsp proteins and the ubiquitin protein targeting pathway initiated by thermal stress; the discovery of a number of carbonic anhydrase isoforms not previously identified in zooxanthellae; the identification of a number of genes coding for carbohydrate metabolising enzymes including those in photosynthesis; the identification of a number of genes coding for proteins involved in nitrogen acquisition and assimilation.

Thermal Stress

With the relentless increase in ocean temperatures as a consequence of global warming it is important to understand the responses of *Symbiodinium*. We have now identified members of the Hsp100, Hsp90 (2 isoforms), Hsp70 protein families along with DNAJ DnaJ (3 isoforms), a Hsp70 co-chaperone, and p23, a Hsp90 co-chaperone (Leggat et al 2005). Previously only Hsp90 and Hsp70 have been characterised from dinoflagellates. We have also identified a suite of proteins essential for the ubiquitin mediated protein degradation cycle. This opens the way to look at expression patterns under a variety of thermal conditions and the differences between clades.

Nitrogen acquisition and assimilation

The relative roles of zooxanthellae and the host in the acquisition and assimilation of nitrogen hashave been contentious. The database has identified a number of the genes encoding crucial transport proteins and enzymes in this process. As a consequence we can now look at the expression of nitrate and ammonium transporters in zooxanthellae, the subsequent reduction of nitrate to ammonium and its assimilation into glutamine by glutamine synthase. This represents a significant advance in our knowledge and provides the capacity to determine how the expression of these genes changes under different nitrogen regimes.

Carbon acquisition, fixation and assimilation

Efficient carbon acquisition and its subsequent fixation underpin the relationship between host and zooxanthellae. An understanding of this process will assist in predicting the consequences of an increase in seawater inorganic carbon concentrations predicted to occur with global climate change. We have previously demonstrated that zooxanthellae possess a carbon concentrating mechanisms (CCM) to overcome the presence of a Form II Rubisco in *Symbiodinium* (Leggat et al 1999). However there is a lack of knowledge on how this CCM operates. The database has revealed the presence of a number of genes encoding carbonic anhydrase isoforms. These sequences will be useful in testing our model for CO_2 acquisition and elucidating the localisation of these enzymes in zooxanthellae. The downstream processes following carbon acquisition can also be investigated as EST database contains a number of the genes encoding enzymes in the utilisation and storage of the fixed carbon eg glyceraldehyde 3-phosphate dehydrogenase.

Conclusions and future directions

The availability of the database is going to be a valuable tool in addressing a number of issues that will contribute to our basic knowledge of this unicellular algal symbiont but also as a powerful investigative tool in understanding how zooxanthellae and the coral symbiosis in general respond to environmental changes. Given the current focus on climate change and anthropogenic effects on coral reefs the bank of information we have can now be applied to understanding how these organisms respond to these challenges.

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Theme 4: Targeted Research Working Group joint field methods (May 22)

Co-Chairs: Rob van Woesik, Yossi Loya

Task: Our task was to assess the dynamics of corals and coral reef communities, identify key process variables, and environmental change under different levels of protection (e.g. MPAs)

Attending**:** Juan Carlos Oritiz, Ove Hoegh-Guldberg, Assaf Zenvoluni, Jeffry Deckenback, Roberto Iglesias Prieto, Ron Johnstone, Mark Davy, Ayax Ruiz Diaz Ruiz, Luisa Falconer, Noa Shenkar, Gidon Winters, Eugenia Sampayo, Paulina Kaniewska, Jez Roff, Tracy Ainsworth, Jessica Gilner, Ranjeet Bhagooli, Peter Ralph, Christian Wild, Baraka Ruguru, Glen Holmes.

Morning session:

1. Introduction to the Coral Reef Targeted Research Working Group joint field methods (R. van Woesik).
2. What are the main ecological questions for the field based studies (generate table).
3. What are the key hypotheses (e.g., Global climate change will alter coral populations to become increasingly skewed toward larger colonies Bak & Meesters, (1999). Am Zoo 39: 56-65) (generate table)
4. What are the key dependent state variables (e.g., coral cover), and process variables (e.g., recruitment rates, mortality rates) that need to be measured (generate table).

Outcomes:

	Key Questions	Hypotheses	State variables	Process variables	Feasibility	Priority
1						
2						
3…						

Afternoon session:

Sampling design and statistical analyses

5. At what spatial scale do we observe the most variance (in for example coral cover)? To what degree does that variance vary among regions and oceans - results of pilot studies (including a short presentation by Juan Carlos Ortiz).
6. Compromises – towards an optimal design.
7. Interpretation and applications (future design modifications).

Tracking coral populations through time

Rob van Woesik[1] & Yossi Loya[2]

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Critical concepts and long-term trends

Recent reports of wide-scale coral bleaching, or paling of corals through the loss of their symbiotic algae (zooxanthellae) and/or their pigments because of stress at and above the corals' acclimation capacity, from all of the world's tropical oceans are a major concern among scientists and resource managers (Loya et al 2001). Clearly, coral bleaching is a global phenomenon linked to global climate change and increasing ocean temperatures (Glynn 1991; 1993; Brown 1997; Hoegh-Guldberg 1999). In the last 2 decades mass coral bleaching events have damaged reef in many localities, yet whether these events cause long term changes to coral communities is unknown.

In 1998, unprecedented worldwide coral bleaching coincided with some of the warmest Sea Surface Temperatures (SST) on record. Coral bleaching was evident from 25°N to 33°N in Japan since nearshore SST in Okinawa were 2.5°C above the ten-year average (Loya et al. 2001). We witnessed another coral bleaching event in 2001 in southern Japan (Van Woesik et al 2004); the thermal anomaly was of similar intensity and duration. In a 2001 publication we showed that some species are tolerant to thermal stresses and will become 'the winners', while others are not so tolerant, and are destined to become 'the losers' (Loya et al 2001). In 2004, we further showed that some coral populations are adjusting to thermal stress events (Van Woesik et al 2004).

Why coral-colony size matters

Many researchers are interested in why some coral species are physiologically more tolerant than others, and why some species will adjust to global climate change. In addition to this we are also interested in whether size frequency distributions (i.e., coral colony size) will be influenced by thermal stress events. Bak & Meesters (1999) proposed that global climate change will alter coral populations to become increasingly skewed toward large colonies. Size-frequency distributions, and relative shifts toward large colonies may have little affect effect on fitness, unless senescence is shown to be a common trait. However, if shifts occur toward small corals, and we know that small juvenile coral colonies are not reproductive, by definition, and neither are most coral colonies that regress back to juvenile size after disturbances, then fitness will be compromised and adaptation unlikely.

We have shown that small coral colonies are more tolerant to anomalous SSTs than large colonies; such small size facilitates mass transfer which in turn aids survival (Loya et al 2001; Nakamura and Van Woesik 2001). This further suggests an increasing trend toward smaller colonies if bleaching events become more frequent. Smaller colonies may not be reproductively competent if they are remnants of once larger colonies, and certainly not when they are new recruits and thus immature. Since the foundation of adaptation is based upon the notion that differential-

reproductive rates are facilitated by environmental influences on different individuals within populations, shifts in size-frequency distributions toward smaller corals maybe a sign that adaptation is less likely to occur if bleaching events are frequent -- because of repeated setbacks toward immaturity.

Community shifts

Of the 1629 colonies examined in southern Japan (van Woesik et al 2004) we recorded colony mortality and assessed the degree of resistance (where no discernible visual change to the colonies were evident) and the resilience among species, depths and colony sizes. While there were obvious differences in accordance with species, there were also differential responses within genera. Coral mortality was more immediate and higher on the shallow reef than on the deep reef, producing a more marked structural shift. Small faviids showed a higher tolerance than large faviids. In May 2002, after 2 thermal-stress events, 720 colonies were recorded (i.e., 44% of those recorded in 1998), which included surviving colonies and new recruits of all 12 species. While the environmental conditions were similarly extreme in 1998 and in 2001, 42% of the coral populations showed an increase in tolerance; more resistant populations included *Pocillopora verrucosa, Porites cylindrica, Pachyseris gemmae, Favia pallida*, and *Favia favus* colonies at two depths (3 & 10 m), which suggest that the populations had adjusted their constitution to the thermal stresses, while *Pavona varians* appeared less resistant in 2001, and the other 6 species, which included *Porites lut*ea, responses remained relatively similar over time. There are only two studies that have reported on coral community shifts associated with 2 bleaching events (Glynn et al 2001; Van Woesik et al 2004). Hence, current information on changes to coral communities following coral bleaching is very limited and there are unclear or controversial projections concerning the long-term effects on the structure of coral communities.

Future directions

Projected climate change may drive temperature and seawater chemistry to levels outside the envelope of modern reef experience. As a consequence, coral reef communities will change. Clearly, juvenile coral colonies are more resistant to a combination of high SST and high irradiance compared with large corals (Loya et al 2001). These results suggest an increasing trend toward smaller colonies if bleaching events become more frequent. Furthermore, we suggest that the relative shifts toward the above-mentioned winners and losers are dynamic, and certainly not static. Therefore, what may appear to be a winning strategy in the short term, through survival of small colonies or the apparent short-term survival of a ‘winning’ growth form, may turn out to be detrimental in the long term, especially if thermal stress events increase in frequency and the time period for colony growth is reduced. Yet, the only means to understand these trends is through long-term assessments of permanent sites.

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Final design for the permanent monitoring program at Heron Island for the GEF-UNESCO-World Bank "Global Coral Reef Targeted Research and Capacity Building Project"

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Coral reefs are characterized as highly dynamic systems through space and time (Veron 1995, Connell *et al* 1997). As a consequence of the rapid deterioration processes affecting coral reefs in the world a better understanding of the way these systems change and the factors affecting these changes is crucial for predicting the future of coral reefs as well as its management (Done 1992, Hoegh-Guldberg 1999, Hughes *et al* 2003).

Most of the ecological monitoring programs target coral cover as the main response variable (Done 1992, Glynn 1994, Connell *et al* 1997). This is a very slow changing variable (Hughes *et al* 2003, Palandro 2003) that should not be rely on its own as an indicator of reef stress since by the time a significant change in coral cover is observed little can be done in terms of management to protect the reef. In contrast, relative abundance of coral species, evenness of coral taxa and specific population dynamics may be much more dynamic variables that could provide managers with earlier alerts about processes affecting the reef (Bak and Meesters 1999, Hughes and Connell 1987, Tanner *et al* 1994, Loya *et al* 2001).

In this project a series of permanent sites will be set on the reef surrounding Heron Island using different sampling techniques within the permanent sites. The main aim of this project is to assess the dynamics of corals and coral reef organisms at the population and community level, and identify key process variables under environmental change

Hypotheses to be tested:

H1 Coral cover, Taxa evenness, relative abundance of coral species, coral recruitment and coral population size frequency distributions will remain constant over time.

H2 Coral taxa evenness responds to climate change at the same speed as coral cover.

H3 Global climate change will alter coral populations to become increasingly skewed toward larger colonies (Bak *et al* 1999).

H4 Population probability of quasi-extinction is constant across different coral species. (Loya et all 2001)

H5 Coral population (x) probability of quasi-extinction is independent of surrounding total coral cover.

H6 Coral population (x) probability of quasi-extinction is independent of surrounding total coral taxa evenness

Methods:

The design explained below is the result of a comprehensive pilot study developed between January and December 2005. The details of this pilot study will be published separately.

Six to seven sites will be established around Heron Reef: 3 to the south, three to the north and potentially (depending on the logistics) one to the east side of the reef (Figure 1). Site is going to be considered as a random factor established systematically. This will be the most important level of information for the analyses.

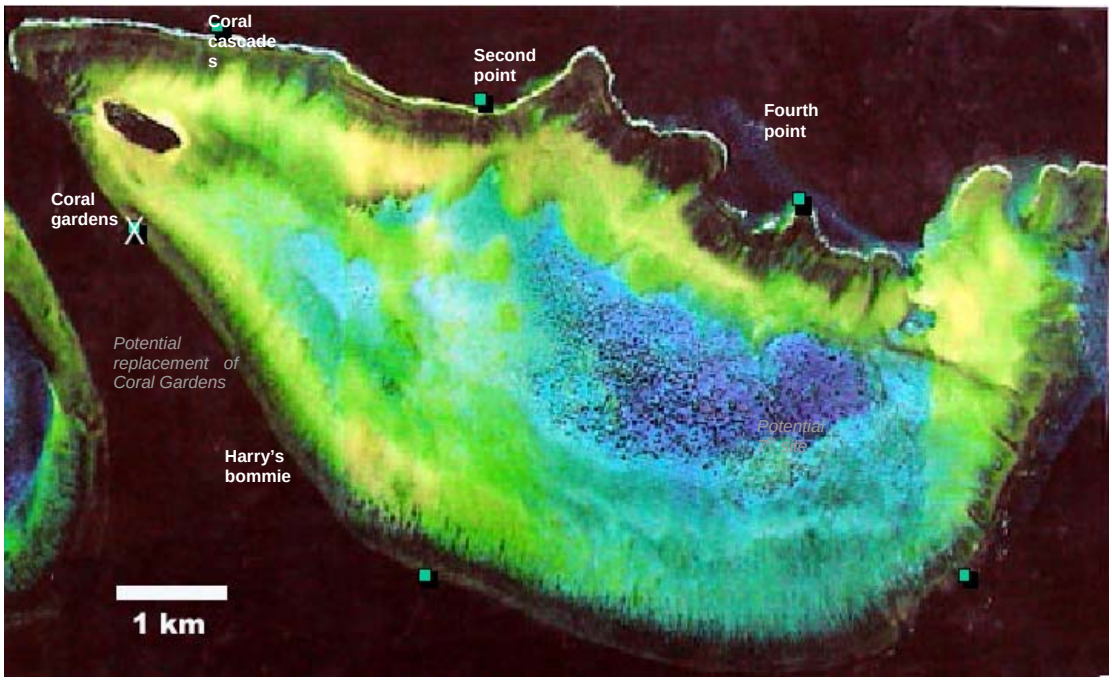

Figure 1: Selected sites around Heron Reef. In white: selected sites, in grey: potential sites

Within each site two sampling areas are going to be fixed. One at 0 meters and one at 5 meters at low tide (reef flat and upper reef slope). Each experimental area will be defined as a 4m wide by 140m long band. Within each sampling area 3 different sampling units will be used:

- 1m x 15m fixed photo transects (n=8). The transects will be fixed systematically in relation to the long axis, and randomly in relation to the short axis. Since on the short axis (4m) there are just 4 potential position to locate a 1m wide transect the randomisation process will be done ensuring that each potential position is used twice.
- 4x4m quadrates (n=3), the quadrates will be fixed systematically along the sampling area (beginning, middle point and end of the sampling area).
- Tagged colonies of targeted species (the species will be chosen after the first set of photo transects has been taken). Between 5 and 10 colonies of each of 5 targeted species will be tagged. The colonies will be selected randomly from the area that is not being photo-sampled within the sampling area. The 5 targeted species will not be the same at both depths due to spatial segregation (most of the species found on he reef flat are different than the ones found on the upper reef slope). The information obtained from the closest transect to each colony will be extrapolated as the surrounded environment for that particular colony. These colonies will be sample each sampling time for physiological parameters. For a schematic presentation of the sampling design see figure 2.

Each site will be sampled every 6 months (unless a big disturbance occurs, in which case extra sampling times will be included).

Information to be obtained from each sampling unit:

Photo transects:

Coral cover, coral community composition (relative abundance of the different families of corals and other substrates). Percentage of bleaching, prevalence of corals affected by diseases that can be detected from the photo, evenness, coral mortality, number of new recruits, etc.

Population size distribution of 4-6 targeted species: These species will be selected ensuring that their abundances, growth form and size range is appropriate to be measured precisely from the photos.

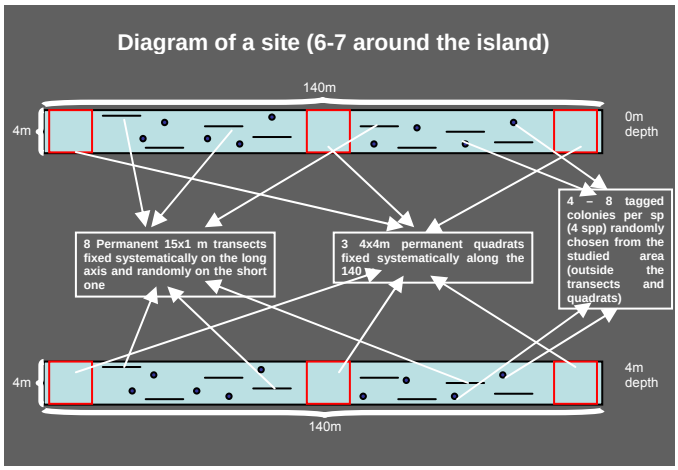

Figure 2: Diagram of an experimental site

Photo quadrates:

Coral cover, composition of coral community (relative abundance of the different coral growth forms and other substrates), percentage of bleaching, prevalence of corals affected by diseases that can be detected from the photo, evenness of morphotypes, and coral mortality. This information will be used to validate remote sensing images.

Tagged colonies:

From the fragments taken: Symbiont density, concentration of photosynthetic pigments, protein concentration, and tissue thickness.

Statistical analysis

The data will be analysed using linear and non-linear regression models. The number of factors and the way each factor will be treated will vary between variables.

“Bulk” variables: The design will allow to perform regression models relating the dependent variables with the covariates as well as make comparisons between sites. A finite population correction will be used to correct for the assumption of all classical statistical analyses that the sampled area is a very small part of the statistical universe, because the proportion of the site area that is being sampled is relatively high (about 20% of the total area of the site).

Physiological variables: The design shown in table one will allow to perform regression models relating the dependent variables with the covariates as well as compare between sites.

Population size distribution: we propose to do an analysis in 3 stages where first we use the mean size per site, then the square root of the variance of the mean size of the site and finally the forth root of the skewness of the size distribution. All this statistics are calculated per site generating a single number for the entire site. Then a regression is done using the values of the six or seven sites trough time.

The only disadvantage with this approach is that in contrast with the analyses done in the previous two sections, this analysis will not allow us to compare between the sites at a specific time, although this comparison is not the main focus of this study.

Using this design we maximise the statistical power of detecting changes in the different variables studied, considering the logistical limitation involved in the sampling. Additional statistical analysis and predicting models could also be applied to further explore more detailed information obtained simultaneously during the sampling.

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Field methods to detect change in the coral communities of south eastern Mexico

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Driving hypothesis

Coral bleaching is a global phenomenon linked to global climate change and increasing ocean temperatures (Glynn 1991; Brown 1997; Hoegh-Guldberg 1999). We are in general interested in which coral species will become 'the winners', while others that are not so tolerant, and are destined to become 'the losers' (Loya et al 2001), and in particular whether size frequency distributions will be influenced by thermal stress events. Our null hypothesis is that global climate change will alter coral populations to become increasingly skewed toward large colonies (Bak & Meesters 1999).

Reef monitoring

Rapid ecological assessments are less concerned about the statistical power of detecting a change than long-term monitoring programs (Andrew and Mapstone 1987). The latter requires appropriate strategies to account for the spatial arrangement of organisms that should yield accurate estimates of coral colony abundance. Besides the spatial considerations associated with data collection, monitoring programs must also decide on the degree of site permanency, and at what level to randomize. A fully randomized design provides characterizations that account for the spatial arrangement of individuals in the communities. High statistical power achieved through randomized designs means that any measured change is indicative of a change in the community, yet sampling effort may need to be tremendously intensive (Green and Smith 1997). It is often convenient to determine geomorphological reef units a priori and sample within those units, instead of identifying high variance among geomorphological units posteriori and then lack the rigor to identify changes over time. A time series of permanent photo-quadrats provides valuable insight into population dynamics, but lacks general information regarding the spatial arrangement of organisms within the community as a whole.

Pilot study

Most pertinent to any long-term monitoring program is the over-riding question: do we have enough information, or statistical power, to detect changes if changes occur? We undertook a pilot study at Puerto Morelos, Mexico, in September 2002 to understand the spatial variance in the distribution of coral communities as a prerequisite for a longer-term monitoring program. Three evenly distributed sites were set up in the back reef habitats in shallow water (~2-3 meters). Sampling was conducted at 3 levels of resolution using replicated: (1) 50 m video transects, (2) 50 m x 2 m belt transects, where we identified each colony to species level and

measured each colony's maximum diameter, and (3) 1 m^2 permanent quadrats, where we photographed each quadrat as a set of 4 x 50 cm^2 images using high resolution digital cameras and assessed each quadrat for coral recruits. Still images were randomly extracted from video tapes and used for analysis. To derive summary statistics we recorded the taxa or substrate type under 10 random selected points per image. Back calculations were made to determine the appropriate sample size required at a 90% confidence level, and suggested that we need a high number of transects to detect a change. Furthermore, we found that an assessment of size frequency distributions of coral colonies using belt transects and permanent sites may be one of the most useful sampling strategies for monitoring.

Proposed monitoring program

We propose to establish at least 10 **Sites** along the eastern Cancun Peninsula of Mexico, which are being subjected to different levels of human disturbance (Fig.1). There will be 2 permanent 50 m by 10 m **Stations** within each site. Three permanent 4 m x 4 m **Quadrats** will be permanently marked within each station. At least 6, 50 m x 1 m **Belt Transects** will be randomly placed in each station and each meter will be photographed with a stereo set of digital cameras mounted on a 1 m^2 frame, with a white color bar attached. We also plan to tag a number of **Colonies** within each Station to examine physiological attributes.

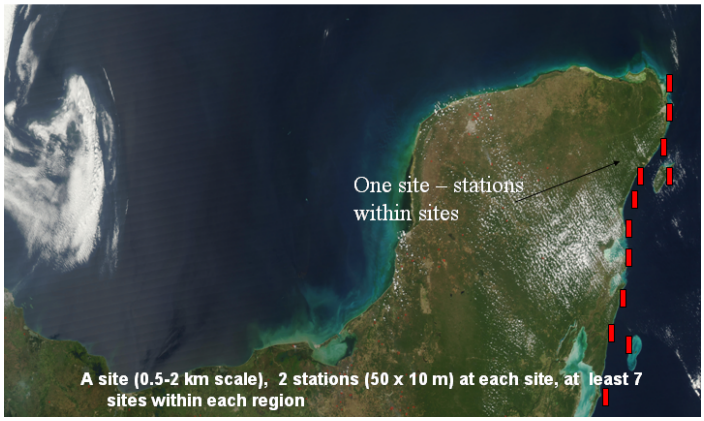

Fig. 1. Proposed study sites along a suspected gradient of human influence on the Cancun Peninsula.

Proposed outcomes

Sites will be examined to assess whether a pollution gradient exists, and whether different coral communities are reflected along that gradient. The advantage of long-term monitoring of permanent quadrats allows the same colonies to be tracked through time allowing elucidation of recruitment, death, and survival rates of the organisms under question. There are some observations in the literature of partial mortality pertaining to stress, but its quantification is limited. This study will elucidate (i) to what degree fragmentation occurs under which conditions and track the fate of the fragments, (ii), what proportion of colonies are new recruits, and (iii) what proportion are survivors. In total, this study will quantify the dynamics of the coral communities, examine size-frequency distributions over time, assess relative shifts in species composition and determine differential colony growth rates, recruitment and post-settlement success. Similar methods will be employed in other regions, for example Heron Island and Zanzibar, which will standardize data and facilitate comparisons.

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Theme 5: Integrated research on coral bleaching and disease (May 24-26)

This workshop was held primarily to explore areas of overlap and synergy between the GEF Targeted Research Groups on Coral Bleaching and Related Ecological Factors (BTRG) and Coral Disease (DTRG).

Discussion conveners/coordinators

John Bythell (j.c.bythell@ncl.ac.uk, Newcastle University)
Drew Harvell (cdh5@cornell.edu, Cornell University)
Ove Hoegh-Guldberg (oveh@uq.edu.au; University of Queensland)

Goals of workshop

The goals of the workshop were to establish important areas of research overlap between the two Targeted Research Groups and to explore areas of potential synergy where integrating or coordinating research activities between the groups will improve outputs. Specific areas considered were:

1. Survey and monitoring of coral populations, disease and bleaching
2. Biomarkers of stress and resistance (="health") in corals
3. Environmental and bacterial causes of coral bleaching.

The initial talks during this section of the workshop included details of planned survey and monitoring of coral populations. The first talk was by Robert van Woesik, which was followed by Ernesto Weil. Discussion then ensued as regard the fine tuning of these field programs to accomplish the ambitions of the Disease Working Group.

Participants of the workshop on "Integrated research on coral bleaching and disease"

Bleaching and physical/chemical stress on coral reefs

Ove Hoegh-Guldberg[1], Michael P. Lesser[2] and Roberto Iglesias Prieto[3]

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Corals loose colour (bleach) when they become stressed, which may arise for a number of reasons including high irradiance or prolonged darkness, excess ultraviolet radiation, low salinity, toxins such as Cu^{2+} or CN^{-}, infection by pathogens such as *Vibrio* and temperatures that are either too high or too low (Hoegh-Guldberg 1999). It may or may not result in the death of corals, an outcome which depends on the extent and length of exposure of corals to a particular stress. Given the key role that corals play in building coral reefs and the fact that recent global episodes of mass coral bleaching have been tied directly to global warming, bleaching has assumed a central importance in our understanding of stress on coral reefs. Despite this importance, our understanding of bleaching is still evolving and during the past several years, several discoveries have caused us to rethink models and perspectives gained over the past 30 years of study. In this paper, we will discuss some of these new ideas and perspectives.

The definition of "bleaching"

Bleaching has been defined by a number of authors as a paling of coral tissues due to the loss of the cells and/or pigments of zooxanthellae (Symbiodinium). While this definition is true in the broadest sense, it fails to incorporate some interesting and important details. Recently Enríquez et al (2005), have shown that the absorptance (the percentage of incoming radiation absorbed) of coral surfaces have a non-linear relation with pigment density. This effect is the result of multiple scattering of light on the highly reflective coral skeleton. As a result of multiple scattering, the optical path length and thereby the probability of absorption by the photosynthetic pigments of the algae is larger in intact coral structures than in isolated cells (see paper by Enríquez, Mendez an Iglesias-Prieto, this volume). The effect of the skeleton on the optical path length is inversely proportional to the density of pigment, which means that the probability of absorption by a pigment decreases as the pigment density of targets increases. This property has an important effect on the perceived colour of a coral undergoing stress. Previous definitions of bleaching have assumed that absorptance varies linearly with the density of symbionts. But was shown in Figure 1, absorptance (under the effect described above) will

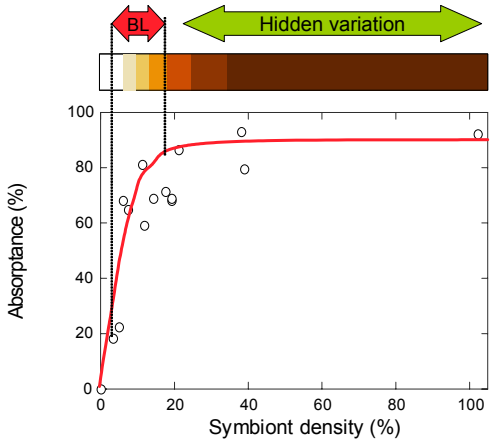

Figure 1. The non-linear relationship between absorptance and symbiont density in reef building corals. BL indicates symbiont density below which visible bleaching begins to occur.

vary asymptotically with the density of symbionts. That is, we will only see a visible effect on the colour of corals (bleaching) in the last stages of any change in symbiont density. The implications of this change are that we are missing much of the story if we only record the colour of corals as perceived by our eyes. While colour is a useful proxy under some circumstances, we need to make careful measurements of the actual density and pigment concentration of *Symbiodinium* in reef-building corals.

The cellular mechanisms of mass coral bleaching

Since 1979, the world's coral reefs have been undergoing an escalating frequency and intensity of mass bleaching events. The latest two examples, the 2005 bleaching event in the Caribbean and the 2006 bleaching event in the Western Pacific are among the most severe bleaching events ever. Mass bleaching events are triggered by warmer than normal seas, which, under sunlit conditions result in symbiotic dysfunction (Brown 1997, Hoegh-Guldberg 1999; Lesser 2004). These changes result in a loss of pigment and/or symbionts, the last stages of which manifest as stark paling of coral colonies. From here the prognosis may be recovery or death of a coral colony, the difference being driven by the intensity of the thermal stress and the length that the corals were exposed to it for. Roughly, corals exposed to low amounts of thermal stress (1-8 degree heating weeks; anomaly size X exposure time, Strong et al. 2000) tended to recover, while those exposed to greater stress for longer (9-14 degree heating weeks) tend to die (Hoegh-Guldberg 2001).

Purely ecological studies of mass bleaching have provided a limited understanding of mass bleaching events, largely due to the lack of an explicit mechanism to explain mass bleaching. In this respect, studies that have focused on defining the underlying mechanism of bleaching have added some very useful information (Hoegh-Guldberg and Smith 1989; Glynn and D'Croz 1990; Iglesias-Prieto et al 1992; Lesser et al 1990, Lesser 2004; Fitt and Warner, 1995; Jones et al. 1998). In this respect, consensus has focused on a model in which bleaching in response to elevated temperature begins with decrease in the photosynthetic activity of the association, most probably associated with a decrease in the ability of *Symbiodinium* sp. to process captured light. Whether this is associated with a lesion close to or down stream from Photosystem II (PSII) is still being debated. However, most evidence points to the dysfunction of PSII leading to an accumulation of oxygen radicals as the energy that normally goes to photochemistry is donated to oxygen via over-excited PSI and PSII components. Under so-called normal conditions, a range of components such as superoxide dismutase (SOD) and ascorbate peroxidase (APO) quench and convert superoxide molecules into less harmful components. However, under high temperature and light, these systems are over-run, with deleterious consequences for cellular components.

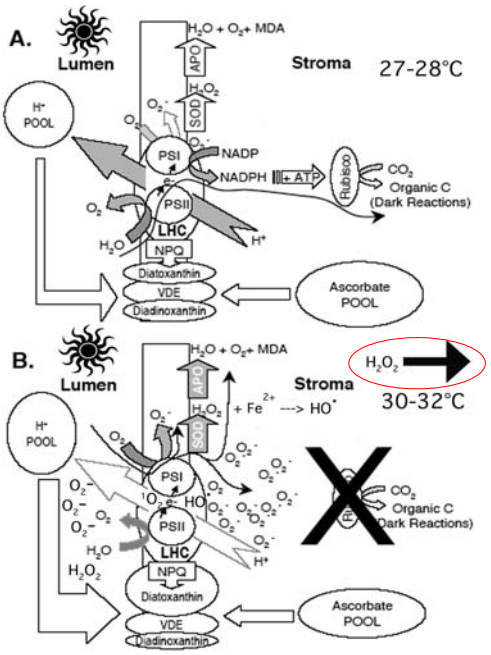

Figure 2. Photoinhibition model for thermal stress related bleaching (adapted from Hoegh-Guldberg 1999)

What happens next is not as clearly defined. Work by Tchernov and others (See Tchernov et al, this volume) has found clear signs that cellular integrity of the algae may play an important role, and that some more resistant varieties of *Symbiodinium* may have more thermally stable thylakoid membrane (TM) lipid compositions. In other work, Tchernov and co-workers have shown that caspase inhibitors will slow the process of bleaching, implying that apoptosis (programmed cell death) may be triggered in coral host cells as they become stressed by active oxygen and other stress related changes. This important insight follows on from the demonstration by Dunn et al. (2002) that bleaching is associated with the activation of apoptosis in *Symbiodinium*. These processes were hinted at in the early 1990s by Gates et al. (1992), and while provocative, require further substantiation. One might summarise the steps leading to bleaching as follows:

1. Environmental stress (physical, chemical or biological change)
2. Excitation processing by PSII is blocked
3. Oxidative stress increases
4. Membranes become dysfunctional
5. Apoptotic processes begin in response to general cellular dysfunction
6. Host cells with algae detach
7. Corals bleach in last stages of process.

Writing these steps down, immediately illuminates how little we do know about the cellular mechanisms of bleaching. The linkage between oxidative stress and membrane dysfunction is still unclear but the mechanism described by Tchernov and coworkers, could be very important in explaining differences between thermally tolerant and susceptible genotypes of *Symbiodinium*. It is also apparent that these studies are heavily focused on the dysfunction of *Symbiodinium*. While *Symbiodinium* is clearly in stress, it would be a mistake to downplay the role of the host. By comparison to the multitude of studies focused on *Symbiodinium*, studies on how thermal stress affects the host are relatively few. It is clear that we need to focus some of the future efforts on the role and susceptibility of the coral host to thermal stress.

One final twist: the role of "photic hell" in escalating stress.

One of the consequences of the extended optical path length within the coral skeletal environment is the enhancement of light fields. As shown by Enriquez et al (2005), irradiance, within the surfaces making up the coral suface, can be up to 4 fold higher than the incidence of light. This, combined with the self-shading effect of pigment targets, means that the light available to a *Symbiodinium* cell within the tissues of a coral may vary many fold depending on the density of *Symbiodinium* within the tissues of the coral host. At high densities of cells, the path length is short and light is efficiently absorbed. At low densities of *Symbiodinium* like those seen in bleached corals, light has a very much longer path length as it bounces around within the highly reflective skeletal surfaces. Under these situations, irradiances can increase many fold above that of the incoming irradiance, as shown experimentally by Kuhl (1995). These light levels may be extremely damaging ("photic hell"), further exacerbating the stress that had originally led to the decline of *Symbiodinium* within the tissues. This, combined with the non-linearity of absorptance against *Symbiodinium* density, may explain the rapidity with which mass coral bleaching events seem to affect coral reefs. Not only are we not aware of the non-visible changes that are occurring within the tissues of coral reefs, but falling densities of *Symbiodinium* led to a dramatic escalation of stress as the efficiency of light absorption is enhanced in the tissues of stressed corals. The resulting positive

feedback loop (less cells ↺ more stress) creates an environment that is extremely hostile to *Symbiodinium*, which leads to the rapid removal of most of the remaining cells. It is clear we need to explore this phenomenon further, especially the role it probably plays in inhibiting recovery. In this regard, understanding this feedback phenomenon may suggest ways that recovery may be enhanced following a thermal event.

Implications for disease

The phenomenon of "photic hell" has implications for any situation in which the density of *Symbiodinium* falls below a particular critical limit. This includes many coral disease-like states in which white patches or areas develop in which pigmentation has disappeared. While it is speculative, the rapid progression of these syndromes may be driven by the extreme photic environments that develop within tissues as pathogens and other influences reduce the population density of *Symbiodinium.* A better understanding of how the consequence of these changes affect the in-tissue light environments is necessary if we are to understand the changes that occur as disease-like syndromes progress.

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Bacterial bleaching of corals

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Vibrio shiloi is the causative agent of bleaching in the coral *Oculina patagonica* in the Mediterranean Sea. Bleaching of *O. patagonica* in the sea occurs only in the summer when water temperatures exceed 26°C. High temperature also plays a key role in bleaching by *V. shiloi* in laboratory aquaria experiments. At 29°C *V. shiloi*-induced bleaching is rapid and complete; below 20°C no bleaching occurs, even with a very high inoculum size of *V. shiloi*. The data indicate that several critical *V. shiloi* virulence factors are produced only at the elevated summer water temperatures, suggesting that primary effect of temperature is on the pathogen, not the host.

Adhesion and chemotaxis

The first step in the infectious process is the adhesion of *V. shiloi* to the coral surface. *V. shiloi* is attracted to the mucus obtained from *O. patagonica*. Motility and chemotactic behavior were present when the bacteria were grown either at 16°C or 25°C. The bacteria then adhere to a β-galactoside-containing receptor on the coral surface. The temperature of bacterial growth was critical for the adhesion of *V. shiloi* to the coral. When the bacteria were grown at the low winter temperature (16-20°C), there was no adhesion to the coral regardless of at what temperature the coral had been maintained. However, bacteria grown at elevated summer temperatures (25-30°C) adhered avidly to corals maintained either at low or high seawater temperatures. The important ecological significance of these findings is that the environmental stress condition (high temperature) is necessary for the coral bleaching pathogen to initiate the infection and become virulent.

Penetration and intracellular multiplication.

Electron micrographs of thin sections of *O. patagonica* following infection with *V. shiloi* demonstrated large numbers of bacteria in the epidermal layer of the coral. Using monoclonal antibodies specific to *V. shiloi*, it was shown that the observed intracellular bacteria were, in fact, *V. shiloi*. The gentamycin invasion assay was used to measure the kinetics of *V. shiloi* penetration into the epidermal cells. The assay relies on the fact that the antibiotic gentamicin does not penetrate into eukaryotic cells. Thus, only *V. shiloi* cells which have penetrated into the coral cells escape the killing action of gentamicin. After adhesion was complete (ca. 12 h), the bacteria began to penetrate into the coral as determined by both total counts and colony forming units. By 24 h, 40-50% of the inoculated *V. shiloi* had penetrated into corals cells. From 24-72 h, the intracellular bacteria multiplied (based on total counts), reaching 3 x 10^8 bacteria per 1 cm^3 coral fragment. When the infected corals were maintained at the high summer temperatures, the bacteria remained at 10^8-10^9 cells per cm^3 for at least two weeks.

Differentiation into the viable-but-not-culturable (VBNC) state

At the same time that *V. shiloi* multiplies inside the coral tissue (24-48 h after infection), the number of colony-forming units (cfu) decreases more than a thousand fold. Entry of bacteria into a state described as VBNC has been reported repeatedly

with a large number of bacterial species. A bacterium in the VBNC state has been defined as “a cell which can be demonstrated to be metabolically active, while being incapable of undergoing the sustained cellular division required for growth on a medium normally supporting growth of that cell”. Intracellular *V. shiloi* cells fit that definition, but unlike most cases of VBNC that have been studied, this is not brought about by starvation or low temperature. Rather, the entry of *V. shiloi* into the VBNC state occurs inside the coral epidermis, where nutrients are abundant.

Toxin P production and mode of action

V. shiloi produces extracellular toxins that block photosynthesis, bleach and lyse zooxanthellae. The toxin responsible for inhibition of photosynthesis, referred to as Toxin P, is the following proline-rich peptide: PYPVYAPPPVVP. In the presence of NH_4Cl, the toxin causes a rapid decrease in the photosynthetic quantum yield of zooxanthellae. The toxin binds irreversibly to algal membranes, forming a channel that allows NH_3, but not NH_4^+, to rapidly pass, thereby destroying the pH gradient across the thylakoid membrane and blocking photosynthesis. This mode of action of Toxin P can help explain the mechanism of coral bleaching. Toxin P is produced at more than ten-fold higher levels at 29°C compared to 16°C.

Role of superoxide dismutase

When corals are infected with *V. shiloi* at the permissive temperature of 28°C, the bacteria adhere, penetrate and begin to multiply intracellularly. If the infected corals are then shifted slowly (0.5°C per day) to lower temperatures, the bacteria die and the infection is aborted. The failure of *V. shiloi* to survive inside coral tissue at temperatures below 20°C is because it does not produce superoxide (SOD) at these low temperatures. This hypothesis was supported by constructing an SOD minus mutant. At 28°C, the mutant adhered to the coral, penetrated into the tissue and then died. Death only occurred when the coral was exposed to light. The most reasonable explanation for these data is that the high concentration of oxygen and resulting oxygen radicals produced by the zooxanthellae during photosynthesis is highly toxic to bacteria and is one of the mechanisms by which corals resist infection. At high temperatures, *V. shiloi* produces a potent SOD which helps it to survive in the coral tissue.

Transmission of the disease

The observations that *V. shiloi* could not be found inside *O. patagonica* during the winter and that the bacterium could not survive in the coral below 20°C indicate that bleaching of *O. patagonica* requires a fresh infection each spring, rather than the activation of dormant intracellular bacteria. Using fluorescence *in situ* hybridization (FISH) with a *V. shiloi*-specific deoxyoligonucleotide probe, it was found that the marine fireworm *Hermodice carunculata* is a winter reservoir for *V. shiloi*. Worms taken directly from the sea during the winter contained 0.6-2.9 x 10^8 *V. shiloi* per worm by FISH analysis. To test if worms carrying *V. shiloi* could serve as vectors for transmitting the pathogen to *O. patagonica*, worms infected with *V. shiloi* were placed in aquaria containing *O. patagonica*. Corals that came into contact with the infected worms showed small patches of bleached tissue in 7-10 days and total bleaching in 17 days. Uninfected worms did not cause bleaching. Thus, *H. carunculata* is not only a winter reservoir for *V. shiloi*, but also a potential vector for transmitting the bleaching disease to *O. patagonica*.

Bleaching of Pocillopora damicornis by Vibrio coralliilyticus

Vibrio coralliilyticus is an etiological agent of bleaching of the coral *Pocillopora damicornis* on coral reefs in the Indian Ocean and Red Sea. Strains of *V. coralliilyticus* have been isolated from diseased corals on the Eilat coral reef, Red Sea and in the Indian Ocean, near Zanzibar. All of these *V. coralliilyticus* strains bleached *P. damicornis* in controlled aquaria experiments. The infection of *P. damicornis* by *V. coralliilyticus* shows strong temperature dependence. Below 22°C no infection occurred. At 24-26°C the infection resulted in bleaching, and at 27-29°C the infection caused rapid tissue lysis and death of the coral. *V. coraliilyticus* produces a potent metalloproteinase at temperatures above 26°C. This enzyme shows high levels of amino acid sequence homology to a range of proteases found in members of the family Vibrionaceae. Because the purified protease caused tissue lysis of corals, it was suggested that at the elevated seawater temperature, where the protease is produced, the bacterium attacks the coral tissues, whereas at the lower temperatures the intracellular algae is the target and the outcome of the infection is bleaching.

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Undergraduate Jez Roff discusses coral disease with Professor Eugene Rosenberg's following his presentation.

Experimental analysis of bacterial ecology of bleaching and disease

John Bythell[1] and Olga Pantos[2]

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The potential interaction between GCC-mediated temperature increase and coral disease prevalence and severity is an area that requires urgent attention. To date, the processes of disease causation and the physiological effects of environmental stress (e.g. bleaching) have been largely studied in isolation, despite some cases of bleaching being directly attributable to pathogens. However, we clearly need a holistic understanding of the sequence of events involved in environmental stress-mediated alterations in disease susceptibility (Fig 1).

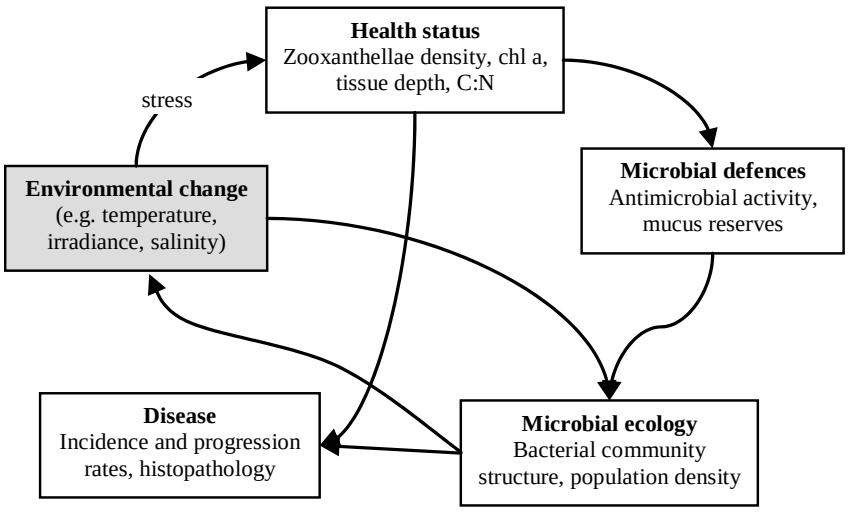

Figure 1. Environmental stress leads to a reduced health status which may be manifest as visible bleaching. The extent to which this stress reduces the coral's antimicrobial defences is unknown. Subtle effects of health status on the microbial community have been shown, but the sequence of alterations in microbial communities versus appearance of disease signs and specific pathogens has not yet been elucidated. Changes in associated microbial community may also promote environmental stress via changes in metabolite production, for example in surface mucus layer biomineralisation processes, leading to a positive feedback loop and potential bacterial proliferation.

There are limitations to using solely traditional culture-based approach to assess these processes. Most (>99%) marine microbes cannot be cultured using standard plate techniques, we know that some coral diseases may be caused by multiple agents (microbial consortia) and diseased and healthy corals are connected by seawater, so potential pathogens are probably everywhere. These factors make it difficult to prove that disease symptoms are unique or that identical symptoms are produced in the laboratory during infectivity studies. Culture-independent methods, including *in situ* localisation of bacteria and sequence analysis are powerful tools for assessing these processes and this seminar outlines studies of known diseases and experimental studies of stress experiments on Heron Island using these approaches. We conclude that a significant area of overlap between the BTRG and DTRG is in elucidating the processes of lesion generation, disease progression and mortality post-bleaching.

Post-mortem Microbial Communities on Dead Corals: Implications for Nutrient Cycling?

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Nutrient cycling (nitrogen, phosphorus, carbon) is an essential part of coral reef ecosystems and is fundamental to their development and survival. While essential to the ecosystem, nutrient loads and associated processes often vary both within and between reefs. These variations also occur over both temporal and spatial scales. Variations from "*mean ambient*" nutrient loads can lead to dramatic ecosystem wide impacts as has been well documented in locations such as Kaneohe Bay, Hawaii and Chapwani, Zanzibar.

Coral bleaching and mortality is becoming an increasingly common and regular event and this increased mortality has the potential to significantly alter the nutrient dynamics within reef ecosystems. Questions that are then raised include: How do the microbial communities developing post-mortem on bleached corals influence nutrient cycling?; What is the significance of changes in microbial community structure?; and; What is the overall significance of such changes for ecosystem functionality at different spatial and temporal scales?

Scale of Influence

Reports of areas of impact following a bleaching event are usually obtained via remote sensing tools such as satellite or aerial imagery. These areas are typically reported as a 2-dimensional area represented by coral colonies and other community members. However, if the 3-dimensional surface area is considered, the actual area of influence at microbial process scale is *significantly* larger. Hence, the functional significance of processes based on these reported surfaces may be currently greatly underestimated; particularly for reefs dominated by highly structured corals.

The challenge then becomes how to sensibly relate micro-scale processes and community performance to the larger scale function and functionality of reefs both spatially and temporally. Aspects of this work include examining community composition, critical conditions for processes (e.g. how much biomass is needed before denitrification can occur – if ever?), succession and the dynamics of these communities/processes under different conditions.

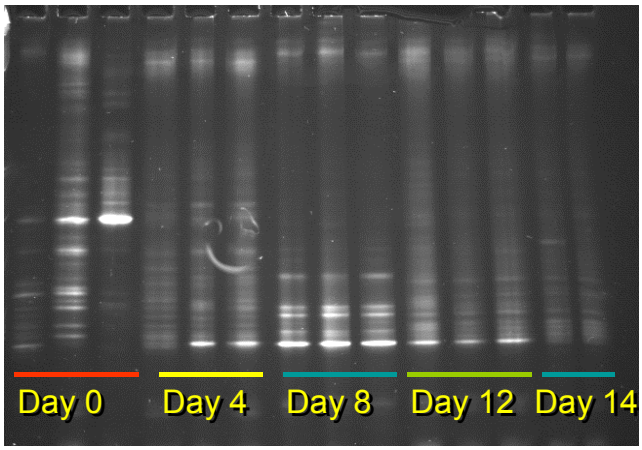

Figure 1: DGGE analysis indicating community structure change over time.

At the microbial scale we know through DDGE and FISH analysis (In prep) that there is a distinct change in community composition and abundance in the days immediately following coral mortality. While results from these analyses are initial, the findings indicate that within the first 10 days of mortality,

the bacterial and cyanobacterial community undergo a series of successions with an increase in overall abundance for a range of species (as would be expected).

At the process level, experiments have thus far concentrated on nitrogen fixation, with present research focussing upon denitrification, nitrification rates ongoing. The initial results based on mortality of *Acropora sp.* show a dramatic increase (approximately an order of magnitude) in the amount of nitrogen fixed per unit area in the 12 days following coral mortality due to thermal stress. When considering the actual surface area involved in mass mortality events (due to bleaching) compared to that reported from remote sensing, the levels of new nitrogen entering the system are therefore likely to be *highly significant* to the ecosystem as a whole.

Overall significance

It is generally accepted that there is a global increase in coral mortality (Wilkinson 2000,2002 & others). For example, in Fiji 40% of corals were lost during the 2000 and 2002 bleaching events. Fiji has approximately 10 000 km^2 of coral reefs. If we assume that 50% of this coverage is live coral, then the mortality following the 2000 and 2002 bleaching events led to approximately 2000 km^2 of potential new surface of nitrogen fixation. If we then consider that this 2000 km^2 is based on a 2-dimensional area, then it is not unreasonable to estimate that this "*new substrate*" is introducing nitrogen into the ecosystem at rates several orders of magnitude above that which would have otherwise been estimated based on background nitrogen fixation rates.

Fiji is not an isolated case. Other areas have reported far greater losses. For example, 4,230 m2 (93%) of *Acropora palmata* and 1,760 m2 (98%) of *Acropora cervicornis* lost at Looe Key, Florida between 1983 and 2000 (Miller et al, 2002).

Conclusions and future directions

The initial conclusions from this work are that the microbial processes occurring in the immediate period following coral bleaching and mortality can have a significant influence on the nutrient dynamics, and therefore function, of coral reef ecosystems. Ongoing work includes:

- Closer examination of microbial functional groups – nitrogen fixers, denitrifiers and nitrifiers;
- The construction of oxygen, carbon, nitrogen, & phosphorous budgets applicable across both temporal and spatial scales;
- An examination of critical states or conditions necessary to support different processes;
- An examination of the influence of the physical complexity of coral surfaces (different coral types) in terms of their microbial community development, type, and process function
- An assessment of the potential for these changes in microbial community to influence other key ecological processes such as the recruitment of primary producing organisms and other functional groups onto newly exposed dead coral surfaces.

Increasing our knowledge in this area will greatly aid coral reef managers in making decisions about what, if any, action that should be taken in order to improve a reefs ability to recover from this increasingly common event.

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Wilkinson CR (2002) State of the Coral Reefs of the World: 2002. Global Coral Reef Monitoring Network (GCRMN). Australian Institute of Marine Science, Townsville

Drew Harvell discusses aspects of John Bythell's presentation to workshop in "Integrated research on coral bleaching and disease".

PAM fluorometry and *Symbiodinium* stress.

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The development of non-invasive techniques based in the detection of chlorophyll *a* (Chl *a*) fluorescence have revolutionize the way we investigate how primary producers respond to different stressful conditions. In the particular case of dinoflagellates in symbioses with invertebrates most of the studies have been motivated by the pressing need to investigate the role played by temperature in coral bleaching. During the last 10 years, the use of these techniques have been instrumental for the study of the mechanisms used by these organisms to cope with stressful levels of several other environmental variables such as light, nutrients and salinity.

The origin of the variable fluorescence

Under physiological conditions, the majority of the Chl *a* fluorescence emitted by the cells is originated at the antenna complexes associated with photosystem II (PSII). PSII is a supramolecular complex that catalyze the light-mediated oxidation of water and the reduction of the plastoquinone pool (Fig. 1). Once light is collected by the photosynthetic pigments, the excitation energy is transferred to the reaction centers were primary photochemistry takes place. Using this excitation energy, one of the two Chl *a* molecules that form the core of the reaction center (P680) is oxidized, reducing a pheophytin which in turn reduces the first stable electron acceptor Q_A. Simultaneous to the formation of the first radial pair at the reaction center, $P680^-$ is reduced by a tyrosine residue located near the core. Ultimately, electrons traveling through PSII are supplied by the oxidation of water. Further down the PSII electron transport chain, Q_A reduces Q_B, which once neutralized by two protons migrates through the membrane to reduce the cytochromes. The intensity of the fluorescence emitted by a sample would be dependent on the redox state of the internal electron transport chain. When Q_A is oxidized, the fluorescence yield is minimal, as most of the energy would be used for moving electrons through the chain. In contrast, when Q_A is reduced, the excitons migrates back to the antenna, producing maximum fluorescence yields. We can use these changes in the fluorescence yield as a sensitive probe of the redox state of the electron transport chain.

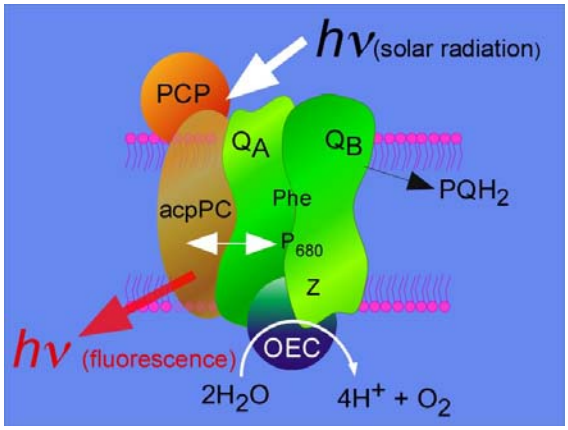

Fig 1. Schematic representation of PSII.

Excitation pressure as a measure of stress

When the rate of primary photochemistry (delivery of electrons) is smaller or equal to the rates of consumption of reducing power in the chloroplasts, the excitation

pressure in PSII is minimal. When the rate of photochemistry is larger than the consumption of reducing power, excitation pressure raises. This condition can be sensed by the photosynthetic apparatus as (i) an over-reduction of the platoquinone pool and (ii) as an increase in the ΔpH between the internal and external parts of the tylakoid membrane. Conditions that result in increases in excitation pressure can be dependent on the light intensity (increases in the rate of light capture), and/or reductions in the rates of reducing power consumption or sink limitations. As a response to the increase in excitation pressure, photosynthetic organisms have developed a series of mechanisms that provide protection against the formation of free radicals that can destroy the membrane. The inductions of these mechanisms can be detected using fluorescence techniques as an increase in non-photochemical quenching (NPQ). These photoprotective mechanisms compete for the Chl *a* excited states with the reaction centers, releasing harmlessly the excess excitation as heat.

Sink limitation as a source of light stress

When cultured *Symbiodinium* cells or intact corals are exposed to elevated temperatures, one of the first detectable responses is an increment in the excitation pressure relative to controls maintained at permissive temperatures. These observations can be interpreted as indicative of a sink limitation, in particular it has been suggested the inactivation of RUBISCO is responsible for the initiation of the events that lead to coral bleaching (Jones et al. 1998). Similar results can be obtained when corals are exposed to other stressors. *Symbiodinium* cells growing actively consume approximately 1/3 of the reducing power generated by the photosynthetic electron transport chain to reduce and assimilate nitrogen. (Rodríguez-Román & Iglesias-Prieto 2005). Exposures to medium without nitrogen or containing enough nitrogen as ammonium to support growth, result in significant increases in the excitation pressure consistent with sink limitation. In general, excitation pressure could be generated when one or more reactions consuming reducing power are either inhibited, or have limitations in the supply of substrate. In this context, exposure to any stressful condition would have synergic effects when it is combined with reduced water flow. The short-term responses to increases in excitation pressure include the induction of the photoprotective mechanisms, and the induction of the PSII repair cycle. If the stressful conditions persist for longer periods of time (days to weeks), a reduction in the optical cross section of the cells is achieved by reducing the concentration of photosynthetic pigments. These reductions effectively reduce the light capture rates in most primary producers. In the particular case of corals, when the symbionts become optically thinner, the multiple scattering of incident light by the highly reflective aragonite skeleton, result in increases in the absorption rates (Enríquez et al. 2005). This effect could explain why small increments in temperature can propagate into the complete collapse of the symbiosis. It is important to notice that bleaching can be the result of exposures to any environmental extreme, and that several signs of several coral diseases include the loss of pigmentation.

Conclusions and future directions

Exposure to a variety of stressors results in increments in the excitation pressure over PSII. From the perspective of the symbiont, any stress would be perceived as an imbalance between the rates of light capture and the use of reducing power. These results suggest a generalized unit of stress in Symbiodinium could be amount of radiation absorbed but not use for photosynthesis. Although the use of PAM fluorometers for the study of stress in *Symbiodinium* has been very fruitful, the use of biochemical techniques to corroborate the results obtained from fluorescence determinations in need.

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Rob van Woesik, Eugene Rosenberg and Baraka Ruguru in discussion during workshop.

Oxidative Stress, Bleaching, and Coral Disease

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Coral reefs are experiencing unparalleled levels of anthropogenically-induced stress. Current estimates on the rate of decline in the health of coral reefs, and the loss or change in community structure of reefs are of worldwide concern (Wilkinson 2000). It is estimated that a combination of physical, chemical and biological stresses will cause the decline of between 40 to 60% of the world's coral reefs over the next 50 years unless appropriate steps are taken (Wilkinson 2000). Until recently, global climate change was seen as just one of many factors (e.g., eutrophication, coastal development, sedimentation, over-fishing) responsible for the decline in the health of coral reefs (Wilkinson 1999) while the time scales of change due to global climate effects was believed to be slow and other anthropogenic causes a higher priority for study. In 1998, however, an estimated 16% of the world's living corals were eliminated in a single warming event related to El Niño (Wilkinson 2000). During this event, sea temperatures warmed to 2-3°C above long-term average summer temperatures and resulted in a catastrophic "bleaching" event that caused significant mortality of several species of coral (e.g., both the expulsion of zooxanthallae and host tissue death occurred). The impact of this thermal event on the percent cover of shallow coral reefs worldwide and the projection of continued rising sea temperatures under greenhouse warming (Hoegh-Guldberg 1999) has radically changed the focus of a large proportion of the research community towards understanding the potential impact of greenhouse driven climate change on the world's coral reefs. Bleaching as a result of thermal stress is not the only threat from global climate change and coral reef biologists from around the world have had to use new experimental tools at all levels of biological organization in their efforts to understand how reefs work, determine which corals will survive anthropogenically driven change, and predict what reefs will look like at the end of the next century. In essence, who will be the "winners" and the "losers" (Loya et al. 2001)?

Exposure to elevated temperatures alone, UVR alone, or in combination can result in photoinhibition of photosynthesis in zooxanthellae. Photoinhibition occurs as a result of the reduction in photosynthetic electron transport, combined with the continued high absorption of excitation energy. One consequence of reducing electron transport is the production of reactive oxygen species (ROS) such as singlet oxygen [$^1O_2$] superoxide radicals [O_2^-], hydrogen peroxide [H_2O_2], and hydroxyl radicals [$OH^{\cdot}$]) for which there are many cellular targets including photosystem II and the primary carboxylating enzyme, Rubisco in zooxanthellae (Lesser 2004). The enzymes superoxide dismutase, catalase, and ascorbate peroxidase act in concert to inactivate superoxide radicals and hydrogen peroxide, thereby preventing the formation of the most reactive form of ROS, the hydroxyl radical, and subsequent cellular damage. Enzymic defenses in the animal host occur in proportion to the potential for photooxidative damage in symbiotic cnidarians. However, high fluxes of ROS in the host or zooxanthellae can overwhelm the protective enzymatic response and result in hydroxyl radical production via the Fenton reaction. Oxidative stress has been proposed as a unifying mechanism for several environmental insults that cause bleaching (Lesser 2004). Oxidative stress can lead to bleaching of zooxanthellae via exocytosis from coral host cells or apoptosis. A cellular model of bleaching in symbiotic cnidarians has been developed and includes oxidative stress,

PSII damage, membrane instability, DNA damage, and apoptosis as underlying processes. This model is consistent with a variety of biomarker proteins expressed in corals during thermal stress.

Damage to photosystem II (PSII) reaction centers in the zooxanthellae, specifically at the D1 protein of PSII, following exposure to elevated temperatures and solar radiation, is believed to be an important factor leading to the bleaching of corals and caused by ROS (Lesser 2004). Damage or impairment of PSII function is easily detected using non-destructive active chlorophyll fluorescence techniques. Instruments have been developed that incorporate protocols to measure the multiple photochemical turnover (pulse amplitude modulated [PAM]), and single photochemical turnover of PSII (fast repetition rate [FRR]) in the laboratory and in the field. These instruments measure, non-destructively, fluorescent transients that provide information on the efficiency of PSII and can discern chronic photoinhibition from dynamic photoinhibition, the former representing damage to PSII and the latter a protective regulatory response of the photosynthetic apparatus. The underwater FRR has been used to examine diel cycling and dynamic versus chronic photoinhibition of corals in shallow and deep waters. One advantage of the FRR versus the PAM instrument is that because of the protocol used to measure fluorescent transients, a series of flashlets that saturate PSII in microseconds this instrument can also measure the optical cross section of PSII which is a valuable parameter for discerning dynamic versus chronic photoinhibition. An underwater version of the PAM instrument is commercially available and has been widely used to study diel changes in the quantum yield of PSII fluorescence and its relationship to differences between photochemical and non-photochemical quenching, or dynamic photoinhibition. Changes in PSII fluorescence have also been correlated with changes in the concentration of D1 protein during exposure to thermal stress and/or solar radiation. Other models of thermally induced bleaching have suggested that the dark reactions of photosynthesis are affected initially, leading to sink limitation, over reduction of photosynthetic electron transport, oxidative stress, and damage to PSII. Lesser (2004) combined the PSII inhibition model with the dark reaction model with oxidative stress as the common effector mechanism for the inhibition of photosynthesis and subsequent bleaching.

One of the most significant changes on coral reefs has been the emergence of diseases and the potential relationship to global climate change. While coral bleaching is most commonly associated with thermal stress and its physiological consequences, bleaching in at least one species of coral, *Oculina patagonica*, is caused by the bacterium, *Vibrio shiloi,* subsequent to thermal stress. A virulence factor associated with bleaching in this coral is the expression of superoxide dismutase by the bacterium that allows it to survive the hyperoxic tissues of the coral. From this observation one could reasonably ask does resistance and pathogenicity in coral diseases related to the modulation of the ROS environment? Do corals depend on hyperoxia and ROS production to defend themselves, and do pathogens have to have, as one of several virulence factors, the ability to detoxify ROS? Is *Oculina patagonica* a better model of coral disease than coral bleaching *per se*? What other virulence factors are essential for successful attachment, penetration, multiplication, and toxin production in a wide range of pathogens and how does the ROS environment affect these steps in the infection process?

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Lunch time discussions with Michael Lesser. Left to right: Michael Kuhl, Mebrahtu Ateweberhan and Michael Lesser.

Seafan epizootic and resistence to Aspergillosis.

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This project is a test of the general hypothesis that compromised immunity drives the Aspergillosis outbreak in seafan corals. This is a plausible hypothesis because *Aspergillus* tends to be an opportunistic pathogen that colonizes immune compromised hosts, for examples humans (*Aspergillus fumigatus*) and insects (*Aspergillus niger*). We are examining the role of climate and environmental factors as facilitators of *Aspergillus* infections. I present evidence for increased temperatures, increasing fungal growth rate, and increased nutrients in the field, increasing lesion severity. We are elucidating specific mechanisms of coral immunity to disease. Our current focus is on peroxidases and chitinases, and I present evidence of anti-fungal activity of both classes of proteins. The seafan- *Aspergillus* pathosystem has been an unusually good experimental system and one future goal is to investigate how general results from this patho-system can be extrapolated to scleractinian-bacterial interactions.

Drew Harvell introduces workshop theme on “Integrated research on coral bleaching and disease”.

Ecology, Physiology and Cell Biology of 'White Syndrome' on the Great Barrier Reef

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Fig 1. WBD affecting *Acropora palmata*. Puerto Morelos, Mexico

During the past two decades there has been a growing concern over the worldwide degradation of coral reef ecosystems (Hoegh-Guldberg 2004) with recent reports estimating a global loss of 27% to date (Wilkinson 2002). Outbreaks of disease-like syndromes have dramatically increased in recent years, with an exponential increase in the number of coral diseases reported since the initial observation of a disease affecting scleractinian coral in 1965 (Sutherland et al. 2004). As emphasised by Richardson (1998), the incomplete characterisation of such 'new' disease-like syndromes in the last decade has caused confusion from not only differing terminology and symptoms for various diseases, but also as to what constitutes a 'disease' (e.g. Borger 2005).

There are at least seven different types of 'white syndrome' that have been reported from the Caribbean region; white plague (WP types I, II & III), white band (WBD type I & II, Fig 1), white pox (WPD) and shut-down reaction (SDR - see Bythell et al. 2004 for review), four of which are known to affect Acroporid corals. Although collectively white syndromes have played a significant contribution to a region-wide decline in coral reefs (Aronson & Precht 2001), pathogens have only been identified within two of these diseases. Whilst white syndromes vary in both rates of lesion progression and patterns of tissue loss (Bythell et al. 2004), they are commonly characterised in the field by distinct signs of tissue loss resulting from the sloughing of the coenosarc, exposing the underlying white skeleton (Bythell et al. 2004), and are likely to represent different etiologies.

The first qualitative descriptions of disease-like syndromes were recorded on the Great Barrier Reef over a decade ago, and several recent studies have documented epizootics in the northern sectors of the GBR (Dinsdale 2002, Jones et al 2004). The results of a five year survey in conjunction with the Australian Institute of Marine Science Long-Term Monitoring Program (Willis et al 2004) has provided a broad overview and insight into novel syndromes and incidence across the GBR, describing several novel syndromes (e.g. brown band, black necrosing syndrome), as well as documenting previously reported diseases with global distributions (e.g. black-band disease). The most notable increase in prevalence was for 'white syndrome', a collective term for symptoms analogous to Caribbean diseases (Fig 2). Distribution

and abundance of white syndrome has dramatically increased over the last 5 years, with a 30 fold increase recorded in the Capricorn Bunker group (southern GBR) between 2002/2003 (Willis et al 2004).

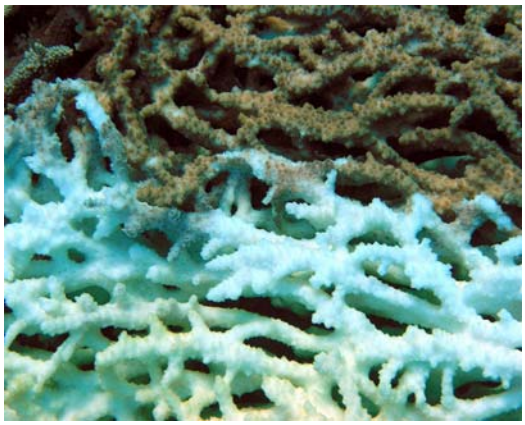

Fig 2. White syndrome affecting tabular *Acropora.* Agincourt Reef, Northern GBR

Our research to date has focused primarily upon the ecology, physiology and cell biology of white syndrome in tabular Acropora spp. from the GBR and has continually emphasised a multi-disciplinary approach to coral diseases. Although significant mortalities have been associated with disease, substantial knowledge gaps exist regarding the ecological processes and interactions across spatial and temporal scales. Kinne (1980) notes that "*diseases affect basic phenomena of life in oceans and coastal waters… in short, diseases are a major denominator of population dynamics*". To gain an understanding of the epizootiology of white syndrome we conducted intensive broad-scale surveys to determine the prevalence of disease at local and regional scales, to describe the spatial distribution of disease-like syndromes and effects of disease upon community structure. Concurrent monitoring of colonies affected by white syndrome was conducted across sites at Heron Reef to determine rates of lesion progression, examine the fate of affected colonies and to elucidate potential interactions between progression of white syndrome and abiotic factors.

The concept of the coral as a holobiont (Rohwer et al 2002) is fundamental to understanding the process of disease and determining the primary target of white syndrome. Our research has investigated the effects of white syndrome on the symbiotic zooxanthellae and phototrophic endolithic communities by investigating both standard physiological parameters and chlorophyll fluorescence using novel tools such as the Imaging-PAM, in collaboration with Karin Ulstrup and Dr. Peter Ralph (University of Technology, Sydney). To further the understanding of the microbial ecology of healthy corals and the shifts in microbial communities associated with disease we have utilised the combination of DGGE as a potential screening tool and sequencing of bacterial 16S rRNA genes in developing clone libraries to investigate key functional groups and identify potential pathogens. At a colony level, the understanding of the host response to white syndrome was examined using ^{14}C labelling to determine the intra-colonial translocation of resources towards progressive white syndrome lesions.

Given previous difficulties associated with culture-dependant techniques in identifying potential pathogens, fluorescent in-situ hybridisation (FISH) in association with confocal laser scanning microscopy was conducted to visualise microbial communities associated with white syndrome. Further investigations utilising histological techniques including in-situ end labelling (ISEL) and haematoxylin and eosin (H&E) were conducted to determine cell death mechanisms and investigate the role of necrosis and apoptosis in white syndrome.

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Towards an Understanding of Coral Disease and Drivers on Indo-Pacific Reefs

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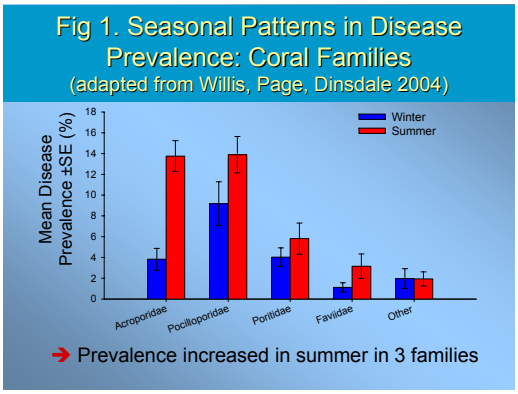
Diseases of coral reef organisms have been escalating in the past few decades, particularly in the Caribbean, but comparatively little is known about the prevalence of coral disease on Indo-Pacific reefs or factors that affect their abundance and severity. A targeted research project funded by the Global Environmental Facility (GEF) seeks to understand the impacts of localized stress and compounding effects of climate change on coral disease globally. As the Australian arm of the WB/GEF Working Group on Coral Disease, we have commenced baseline surveys of coral disease in the northern, central and southern sectors of the Great Barrier Reef (GBR) Marine Park as a first step in understanding the epidemiology of coral disease in the region.

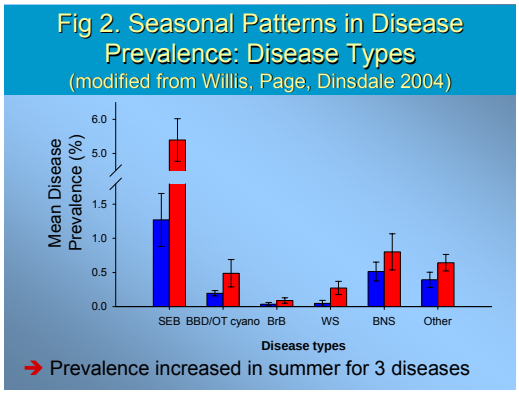
We recognise seven disease states (tumors, skeletal eroding band (SEB), black band disease (BBD), other cyanobacterial syndromes, white syndrome (WS), brown band (BrB), and atrementous necrosis) that affect scleractinian corals and one that affects gorgonians. White syndrome has been introduced as a general category for disease states on Indo-Pacific reefs that manifest as an area of recently exposed white skeleton adjacent to healthy or bleached tissue. There could be a variety of causes or triggers for the apparently rapid tissue loss associated with the syndrome, including a variety of pathogens associated with the Caribbean white diseases or cellular mechanisms associated with stress events. Brown band is a new disease first reported from the GBR (Willis et al. 2004). Six of these disease states (SEB, WS, BBD, other cyanobacterial syndromes, BrB and tumors) were widely distributed, being found in all sectors and associated with at least three of the more abundant coral families (Acroporidae, Pocilloporidae, Faviidae). Thus, disease is a natural part of the ecology of coral populations on GBR reefs.

Seasonal patterns in disease prevalence

Disease prevalence increased dramatically between winter and summer surveys on reefs in the far northern sector, more than doubling in the acroporids and faviids (Fig 1). In particular, the number of cases of SEB, BBD and WS was greatest in the austral summer (Fig 2), suggesting a link between higher temperatures and disease incidence for these syndromes. In contrast, BrB prevalence did not differ significantly between seasons over two years and experimental studies found no impact of elevated temperatures on rates of progression across host colonies. We conclude that coral pathogens vary in their response to temperature within seasonal ranges on the Great Barrier Reef. However, given putative links between disease prevalence and elevated temperatures for the majority of diseases surveyed, plus current trends in global climate change and intensity of human-related activities that compound stress in corals, studies such as ours on the Great Barrier Reef are critical for

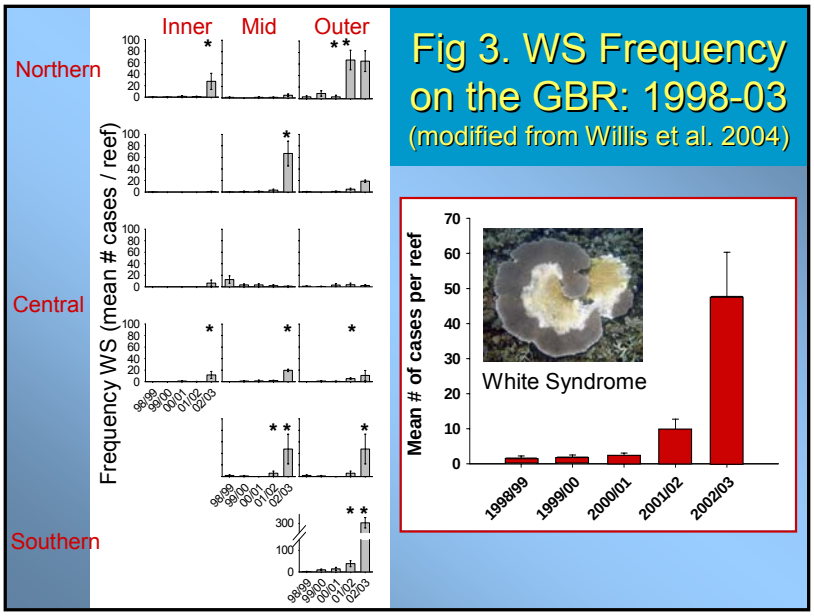
establishing global baselines against which to judge whether background levels of coral disease are increasing.





Coral disease outbreaks: the importance of diversifying approaches to understand the impacts of disease on coral populations

A number of outbreaks of coral diseases have been detected on Indo-Pacific reefs in the last 5 years, including an outbreak of white syndrome cases in 2002/03 on outer-shelf reefs in the northern and southern sectors of the GBR (Fig 3), a white syndrome outbreak on Marshall Island reefs in 2003/04 (D. Jacobsen, pers. comm.) and a white syndrome outbreak on reefs in Palau in 2005 (GEF Coral Disease Working Group, unpubl. data). In the latter 2 cases, surveys of disease prevalence did not detect the outbreaks, highlighting the need for a research approach that combines quantitative surveys of disease prevalence with qualitative reconnaissance surveys and monitoring of tagged colonies in order to understand the impact of disease on coral populations.



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