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

Peter Ralph

Department of Environmental Sciences, University of Technology, Sydney
Cnr Westbourne Street & Pacific Highway, GORE HILL NSW 2065

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.*

Roberto Iglesias-Prieto

Unidad Académica Puerto Morelos, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Apartado Postal 1152, Cancún QR 77500, México.

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.

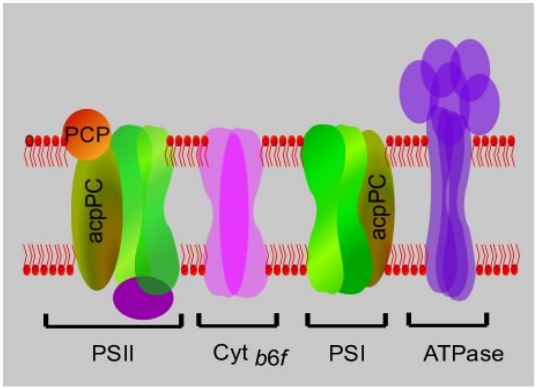

Fig 1. Schematic representation of the photosynthetic apparatus of *Symbiodinium*.

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

Susana Enríquez, Eugenio R. Méndez[1], & Roberto Iglesias-Prieto.

Unidad Académica Puerto Morelos, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Apartado Postal 1152, Cancún QR 77500, México; 1. Departamento de Óptica, División de Física Aplicada, Centro de Investigación Científica y Educación Superior de Ensenada, Km 107 carretera Tijuana-Ensenada, Ensenada BC 22860, México.

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

Mark E. Warner[1]

1. University of Delaware, College of Marine Studies, 700 Pilottown Rd. Lewes, Delaware, 19958, USA.

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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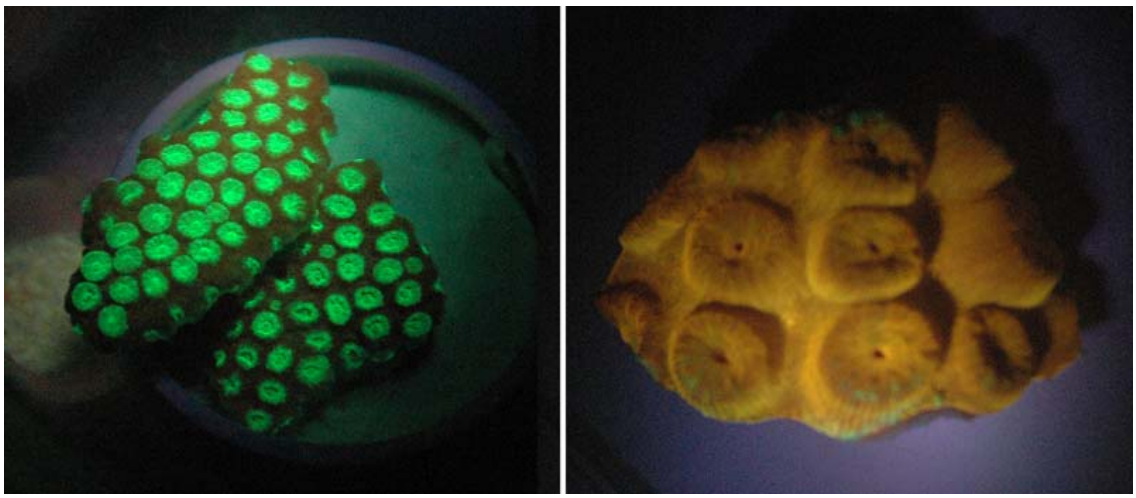
Diel Cycling of Nitrogen Fixation in Corals with Symbiotic Cyanobacteria

Michael P. Lesser[1], Luisa I. Falcón[2], Aimé Rodríguez-Román[3], Susana Enríquez[3], Ove Hoegh-Guldberg[4], and Roberto Iglesias-Prieto[3]

1 Department of Zoology and Center for Marine Biology, University of New Hampshire, Durham, New Hampshire 03824, USA; 2 Instituto de Ecología, Universidad Nacional Autónoma de México, Circuito Exterior s/n Ciudad Universitaria, CP 04510 México, D. F. México, 3 Unidad Académica Puerto Morelos, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Cancún QR 77500, México, 4 University of Queensland, Centre for Marine Sciences, 4072, St. Lucia, Queensland, Australia

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



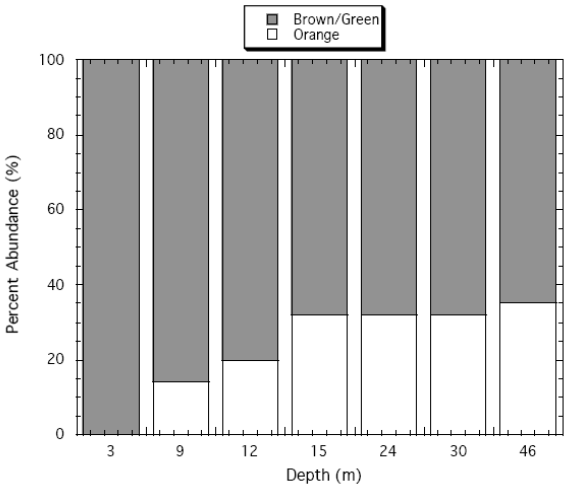


Figure 2 Percent abundance of Montastraea cavernosa colonies containing endosymbiotic cyanobacteria at different depths around Lee Stocking Island, Bahamas. Significant differences were observed with depth using a contingency table analysis; Likelihood ratio, $\chi 2$ =18.17 P=0.006, Pearson, $\chi 2$ =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.

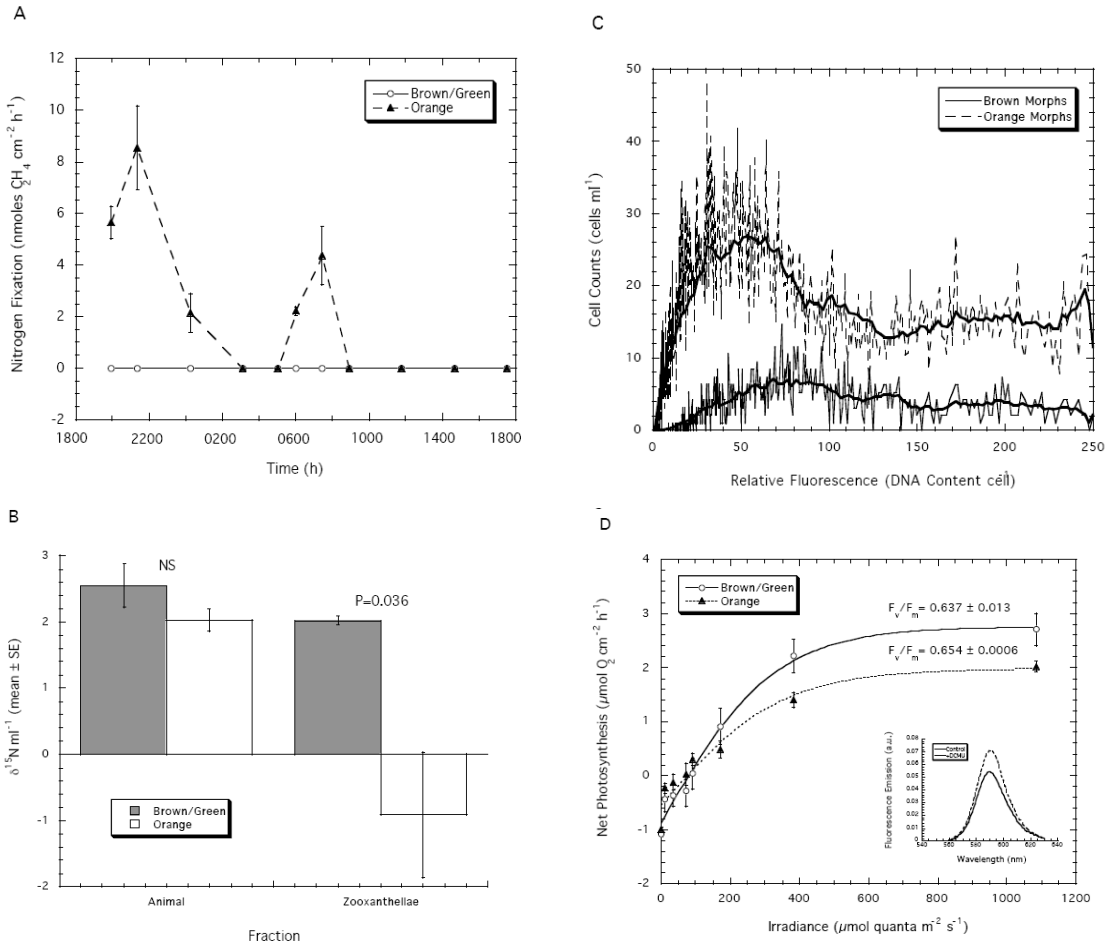


Figure 3. a) Nitrogen fixation (acetylene reduction) brown/green (N=3) versus orange (N=3) colonies of M. cavernosa. There were significant effects of colony type (ANOVA: P<0.0001), time (ANOVA: P<0.0001), and the interaction of colony type and time (ANOVA: P<0.0001), Post-hoc multiple comparisons show 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.

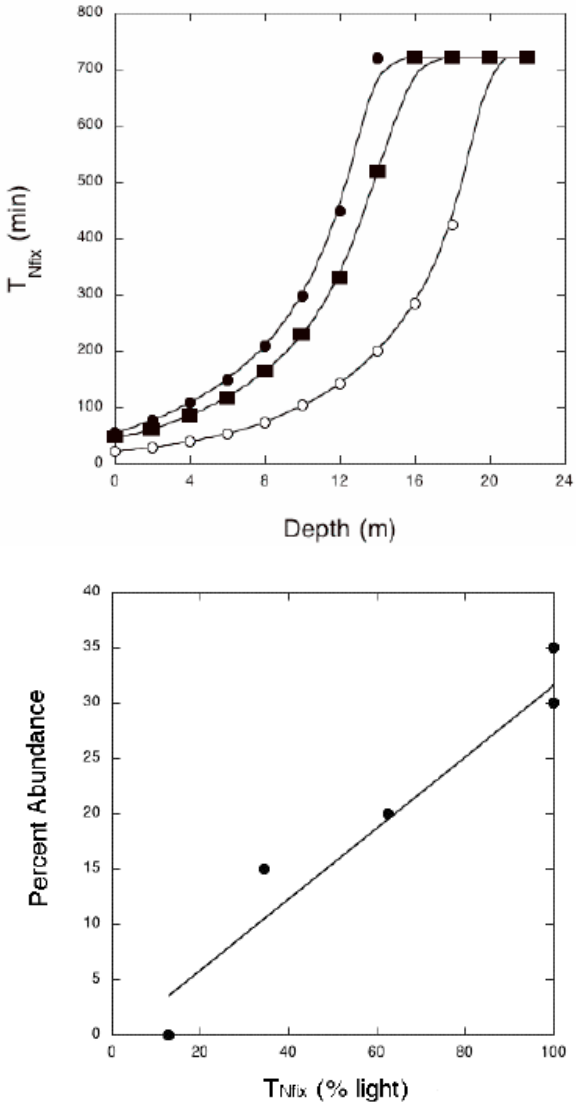


Figure 4. a) The relationship between time (min) and depth (m) during which nitrogen fixation can take place in *Montastraea cavernosa* with endosymbiotic cyanobacteria. The open circles represent predictions using the data from the fitted P-I curve. The closed squares are the simulation results using 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

Sophie Dove[1], Carli Lovell[1], Maoz Fine[1], Susana Enríquez[2], Roberto Iglesias-Prieto[2], Kenneth Anthony[3] and Ove Hoegh-Guldberg[1].

1. Centre for Marine Studies, University of Queensland, St Lucia 4067 Australia; 2. Unidad Académica Puerto Morelos, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Apartado Postal 1152, Cancún QR 77500, México; 3. School of Marine Biology and Aquaculture, James Cook University Townsville 4811 Australia.

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.

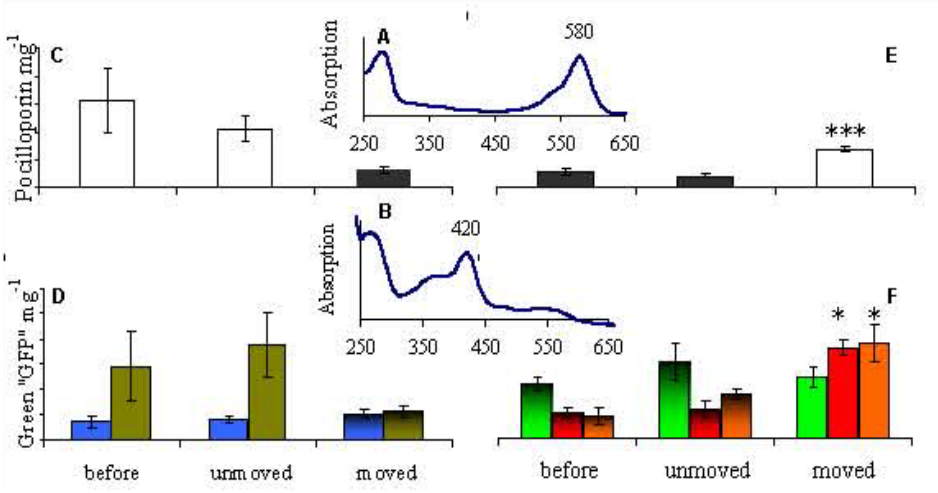


Figure 1. Effect of transplanting *Montipora monasteriata* between high (unshaded) and low (shaded) light fields on host pigments. Absorption spectrum of (**A)** pocilloporin (**B**) putative green GFP. Transplantation of blue and tan morph from high to low PFD **(C,D)**; of green, red and brown morphs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

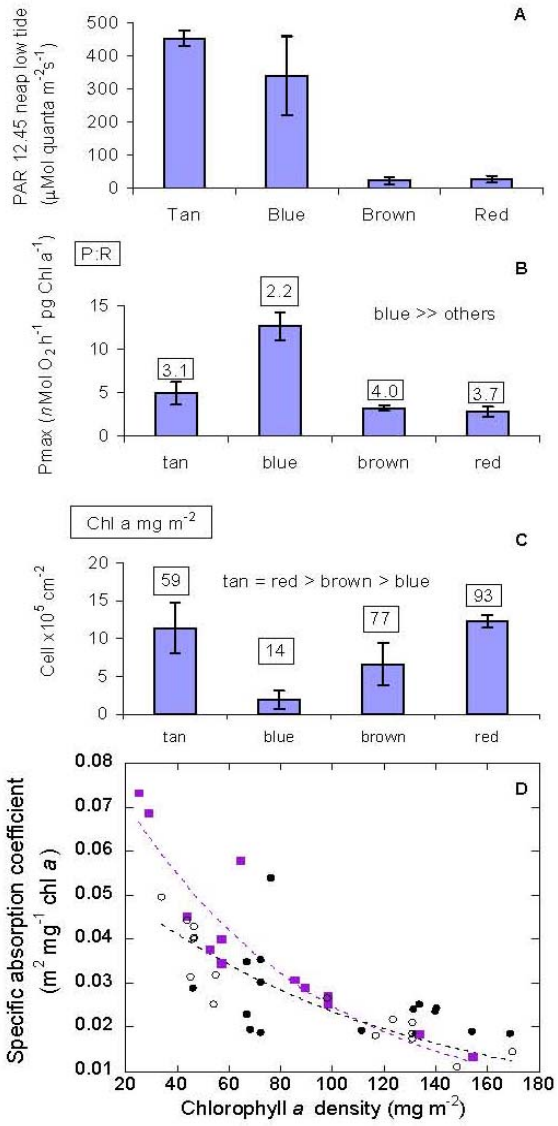


Figure 2. Typical mid-day light fields (**A**); maximum rate of photosynthesis; boxed text shows ratio of photosynthesis to respiration (**B**); areal algal cell densities; boxed text shows corresponding Chl *a* density (**C**); estimate of algal light absorption (*a**) relative to Chl *a* density for 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

Ross Hill[1] and Karin E. Ulstrup[1]

[1] Institute for Water and Environmental Resource Management and Department of Environmental Sciences, University of Technology, Sydney, Westbourne St, Gore Hill, NSW 2065, Australia.

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}.

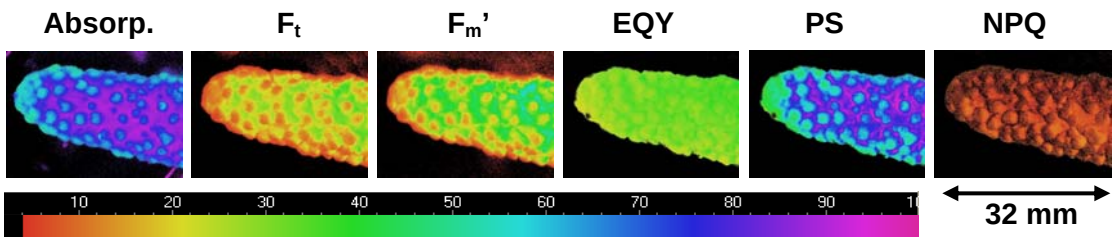

Fig. 1: Chl *a* fluorescence images of *A. nobilis* showing PAR absorptivity, F_t, F_m', effective quantum yield (EQY), relative photosynthesis rate (PS) and non-photochemical quenching (NPQ). Colour scale shown.

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*.

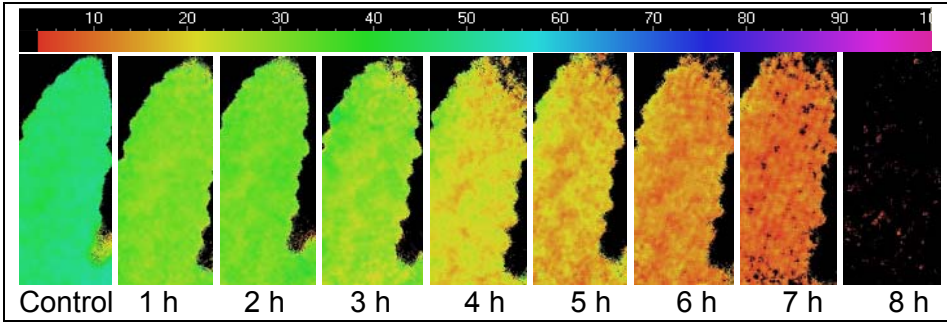

Fig. 2: EQY of *A. nobilis* over 8 h of exposure to bleaching conditions. Colour scale shown.

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.

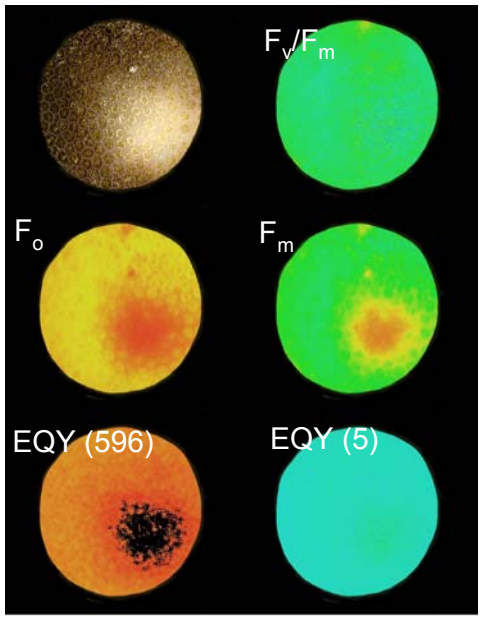

Fig. 3: Photograph and variable chlorophyll *a* fluorescence of *Porites sp.* showing F_v/F_m, F_o, F_m, EQY at 596 and 5 µmol photons m^{-2} s^{-1}.

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*

Gidon Winters[1], Sven Beer[1*] and Yossi Loya[2]

[1]Department of Plant Sciences, Tel Aviv University, Tel Aviv 69978, Israel; Department of Zoology, Tel Aviv University, Tel Aviv 69978, Israel

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

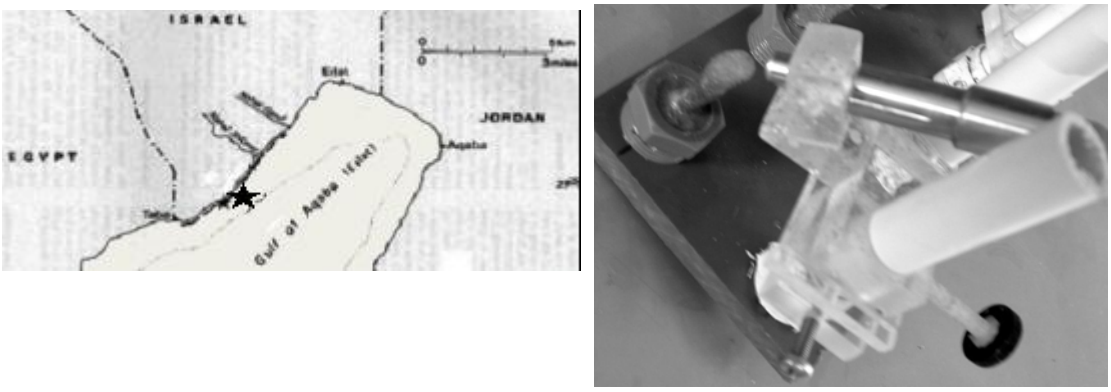

Fig.1. a) Study site and b) experimental set up: Specially made plastic holder for the Diving-PAM's probe, allowing for repetitive measurements to be performed keeping the same angle (69o) and distance (1 cm) between the sample and the PAM's probe

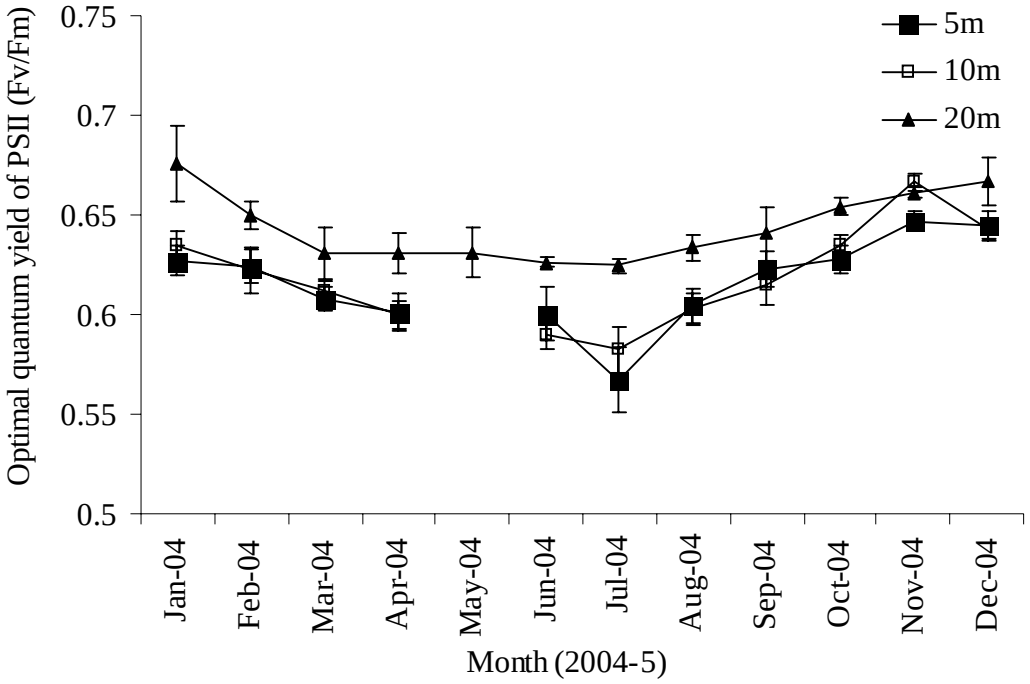

Fig. 2. Seasonal variations in F_v/F_m in *Stylophora pistillata* (n=5, ±SE) growing at 5, 10 and 20m

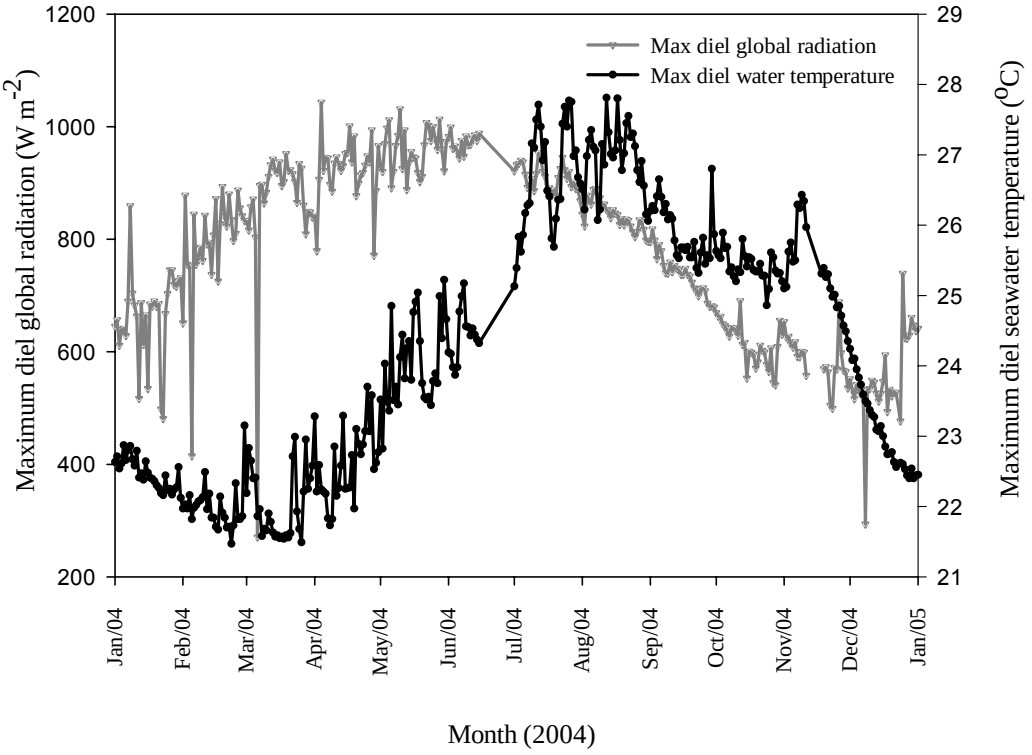

Fig. 3. Seasonal variations in global diel maximum global radiation (left y-axis) and diel maximum seawater temperature (right y-axis) for the year 2004.

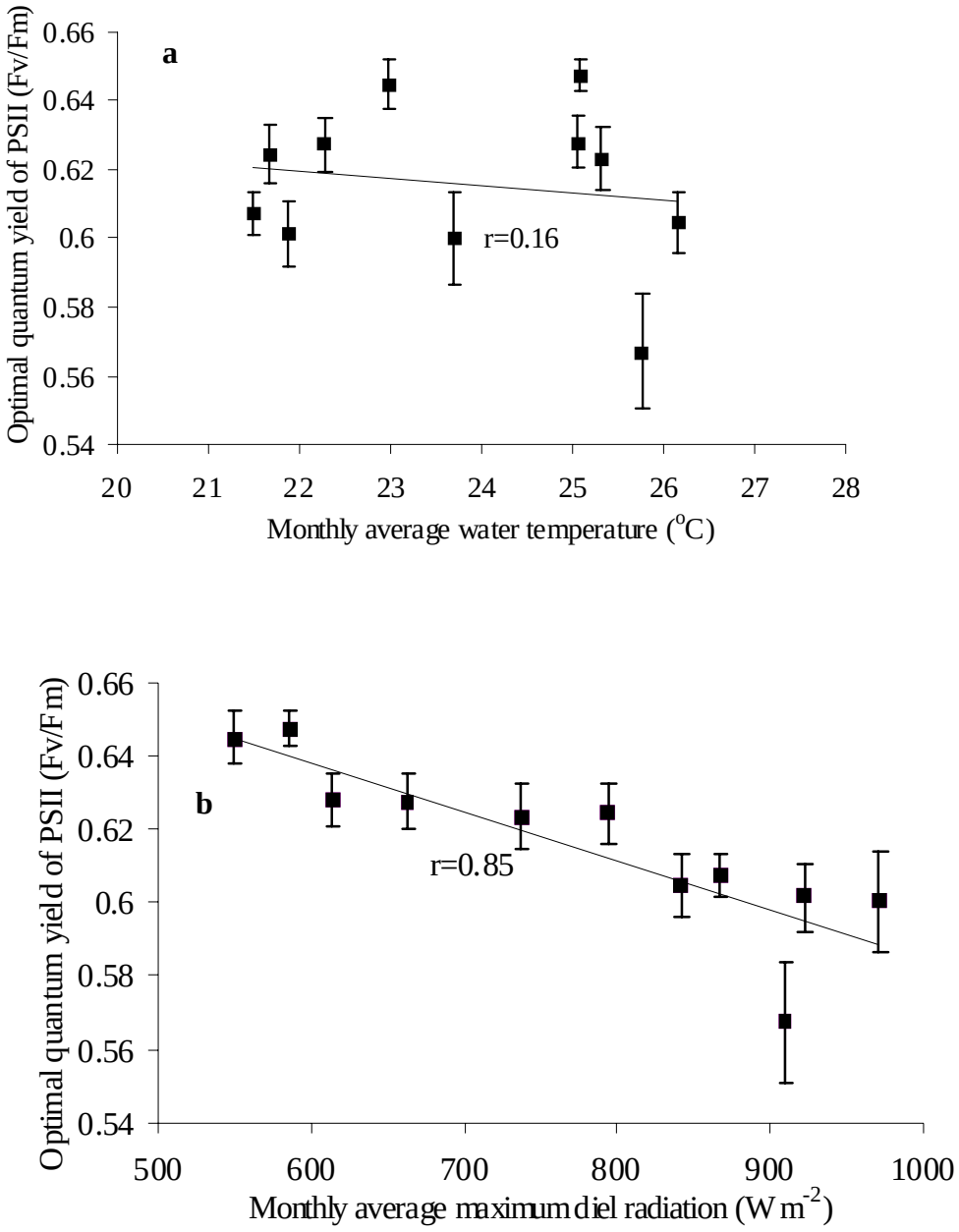

Fig. 4 Correlations between monthly F_v/F_m measurements of *Stylophora pistillata* (n=5) growing at 5m and monthly average of a) seawater temperature (°C) and b) diel maximum global radiation (W m^{-2})

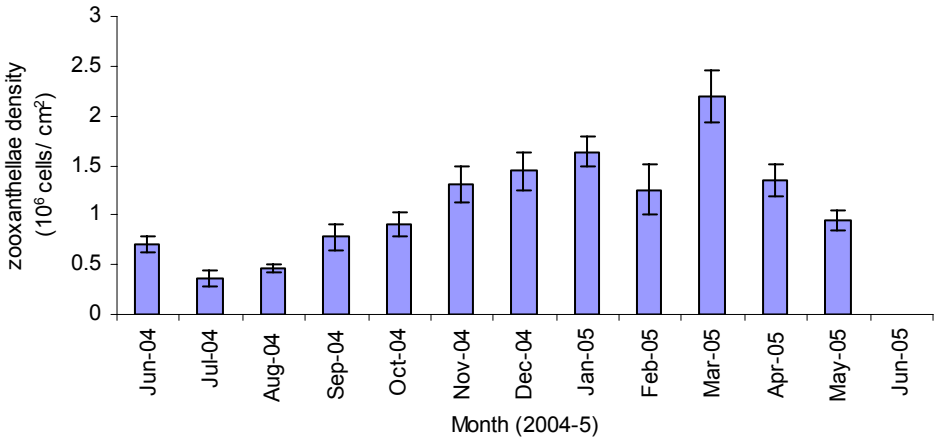

Fig. 5. Seasonal dynamics in zooxanthellae density of *Stylophora pistillata* (n=5-6, ±SE) growing at 5m

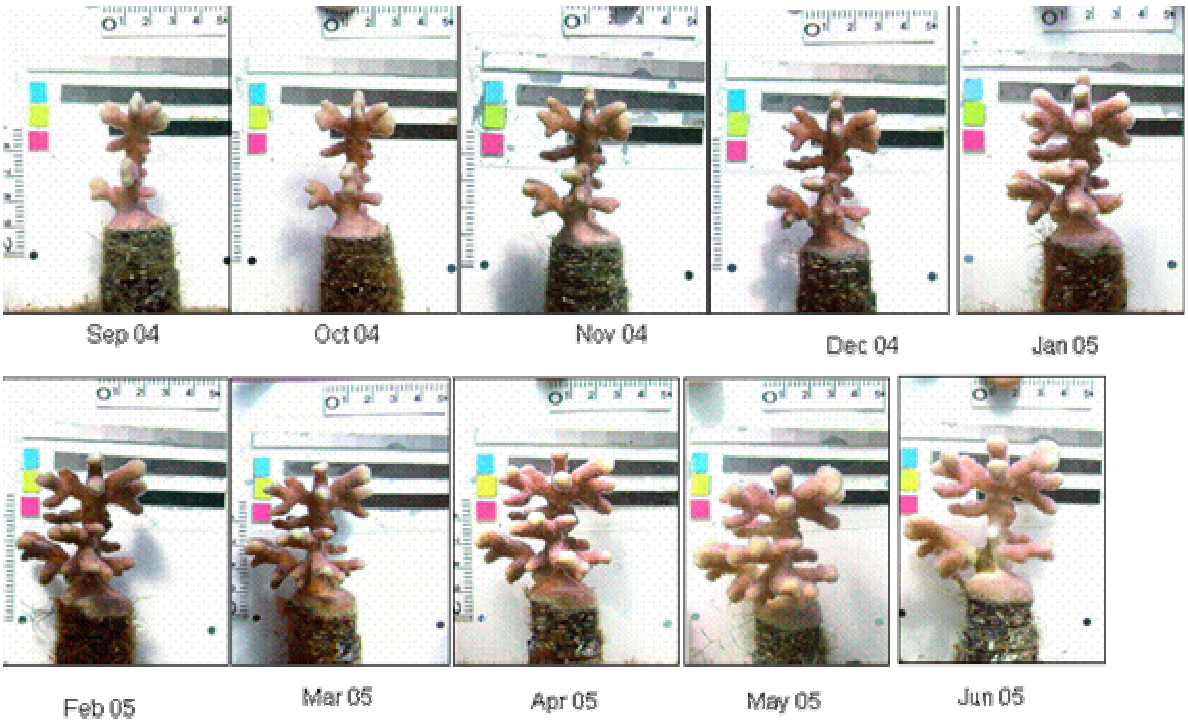

Fig. 6. Photographs following the same branch of *Stylophora pistillata* growing at 5m through out the year.

The cellular mechanism of coral bleaching

Daniel Tchernov [1,3] , L Haramaty[1], T. S. Bibby[1], Max Y. Gorbunov[1],and Paul G. Falkowski[1,2]

1 Environmental Biophysics and Molecular Ecology Program, Institute of Marine and Coastal Sciences, Rutgers University, 71 Dudley Road New Brunswick, NJ 08901,USA; §Department of Geological Sciences, Rutgers University, Wright Geological Laboratory, 610 Taylor Road, Piscataway, NJ 08854, USA; ¥ The Interuniversity Institute of Eilat, P.O.B 469, Eilat 88103, Israel

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.