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RAPID: A Unique Cruise Opportunity to Test the Effect of Trace Metal Limitation on Oxidative Stress and Coral Bleaching

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Wells, Mark - Principal Investigator

Title:

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Project Participants

Senior Personnel

Name: Wells, Mark

Worked for more than 160 Hours: Yes

Contribution to Project:

Name: Shick, J. Malcolm

Worked for more than 160 Hours: Yes

Contribution to Project:

Name: Trick, Charles

Worked for more than 160 Hours: Yes

Contribution to Project:

Dr. Charles Trick has been involved in the coral oxidative stress project from the beginning, as both a 'co-PI', and laboratory and field studies participant.

Name: Dunlap, Walt

Worked for more than 160 Hours: Yes

Contribution to Project:

Dr. Dunlap was our collaborator at the Australian Institute of Marine Sciences, and was critical to success of the project by providing his expertise, laboratory facilities and cruise opportunities.

Post-doc

Graduate Student

Undergraduate Student

Technician, Programmer

Other Participant

Research Experience for Undergraduates

Organizational Partners

University of Western Ontario

Dr. Trick has been a key collaborator on the project, providing both students and equipment essential to success of the project.

Other Collaborators or Contacts

Activities and Findings

Research and Education Activities:

This project was to augment an existing project to enable us to participate in a final cruise to the Great Barrier Reef that provided us a necessary comparison dataset. It is functionally not possible to separate the research and education activities of the two grants. For that reason we have submitted the completed same final report for the combined studies to both this grant and the Main project.

See attached file

Findings:

See Attached File

Training and Development:

See Attached File

Outreach Activities:

See Attached File

Journal Publications

Books or Other One-time Publications

Web/Internet Site

Other Specific Products

Contributions

Conference Proceedings

Categories for which nothing is reported:

Any Journal
Any Book
Any Web/Internet Site
Any Product
Any Contribution
Any Conference

Final Report

Effects of Trace Metal Limitation on Oxidative Stress in Zooxanthellae and its Role in Coral Bleaching

Major Research and Educational Activities:

This report briefly summarizes the major findings and accomplishments of investigations of the effects nutrient trace metals have in modulating the oxidative stress of corals and their endosymbiotic dinoflagellates (zooxanthellae). The project has had significant successes and marked failures, the latter primarily due to the difficulties in rearing and maintaining healthy corals in trace metal regulated conditions. Our effort included both major laboratory experiments and shipboard field incubations during 6 short cruises to the central region of the Great Barrier Reef off Townsville, Australia, allowing us to study both summer and fall conditions. The project received strong support, both financial and logistical, from the Australian Institute of Marine Sciences, Townsville, Australia, without which the study would not have been possible. The AIMS-funded research cruises were vital to success of the project, both in terms of enabling field sampling to assess metal distributions and to characterize the typical diurnal photophysiological signals of healthy *Stylophora pistillata* in offshore and nearshore environments. They also provided the key role of coral collection for the laboratory experiments. We wish to express our sincere thanks for the generosity and good will uniformly expressed to us by the AIMS staff and scientists.

The project had three major thrusts, all centered on assessing the effect of trace metal deficiency, or small augmentation with required trace metals, on the health of *S. pistillata*, an abundant and well-studied coral species in these waters. The first was to characterize the distribution of dissolved Fe and Cu; two key trace metals used in antioxidant defenses (Fig. 1) and known to be limiting in some oceanic environments, across the GBR from nearshore to the outer reef margin. Second, we investigated the effect of Fe and Cu addition and removal from seawater on the ability of *S. pistillata* to respond to high light and high temperature conditions, both in the laboratory and in field incubations. The key signals used to assess coral response were photophysiology (measurements of variable fluorescence) and antioxidant enzyme activity, as well as the general appearance of the coral test nubbins. Mn also is an important metal in antioxidant systems, but it was not studied here because 1) dissolved Mn concentrations remain elevated in even remote tropical surface waters (Obata et al. 2008), 2) it is difficult to regulate its biological availability independent of other metals, and 3) the logistical demands of testing a third metal under controlled light fields was beyond our laboratory capabilities. In our third thrust we used exploratory gene expression and proteomics analyses to evaluate the coral and endosymbiont responses to combined light, temperature, and trace metal stress conditions.

One of the central achievements of the project was to successfully develop novel techniques for studying trace metal limitation of corals including the fabrication, testing, and implementation of entirely new culture vessel designs for experiments on corals under conditions in which trace metals are controlled. Our initial studies showed that

corals were extraordinarily sensitive to synthetic seawater media used for studying the trace metal requirements of marine phytoplankton, with sudden and complete collapse of

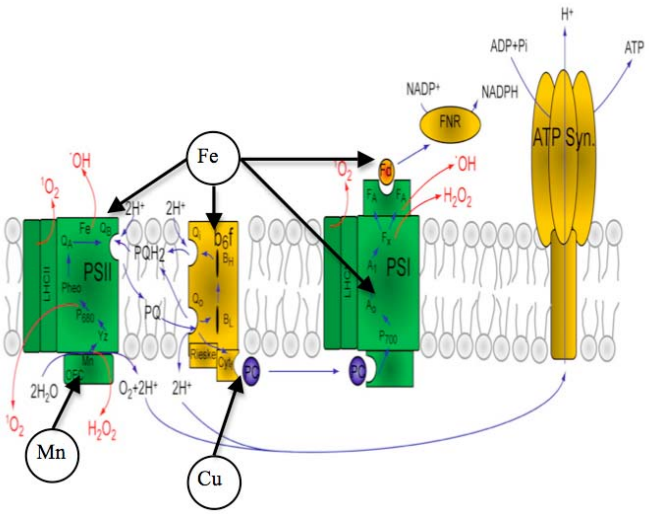

Figure 1. Diagram of the photosynthetic electron transport chain, ROS generation and trace metal requirements, modified from Shcolnick and Keren (2006). Highlighted within the diagram are the locations of Cu, Fe, and Mn within the photosynthetic electron transport chain. Cu is a critical component of plastocyanin (PC), a molecule responsible for electron movement between cytochrome b6f and PSI (Shcolnick and Keren 2006). Fe plays structural roles within PSII, the cytochrome b6f complex, PSI and the protein ferredoxin (fd) and four Mn ions are responsible for the transfer of electrons from water to PSII (Shcolnick and Keren 2006).

the colonies within 5 days despite frequent media changes. Therefore, we opted to strip metals from natural seawater by combining UV oxidation to break down metal-complexing ligands, followed by ion exchange removal of the free metals. This medium extended coral survival for at least three weeks, and probably longer. The second challenge was to develop the methodology for maintaining trace metal clean conditions during incubations. Standard coral maintenance methods using flowing seawater baths was not possible given the very large volumes of prepared media that would be required. We instead fabricated closed polyethylene containers equipped with filtered-air bubblers and coral hangers that allowed extended coral survival with daily media exchange. These sealed containers we immersed in temperature controlled flowing seawater baths under regulated light fields. For sampling and media exchange, the containers were transferred to a Class 100 clean bench within a positive pressure mini-lab fabricated within a larger conventional laboratory. The same containers and clean room sampling strategy were used during the research cruises. Tests showed that these methods prevented trace metal contamination of the corals during our experiments.

Major Findings:

Effect of Iron Limitation on Coral Colonies at High Temperature: Our findings show that low iron availability in seawater can compromise photosynthetic efficiency of zooxanthellae *in hospite*, suggesting that iron limitation may facilitate chronic photoinhibition at elevated temperatures. We increased and decreased metal availability

in laboratory incubation studies using colonies of the coral *Stylophora pistillata* collected from inner regions of the Great Barrier Reef (GBR). Corals for these laboratory experiments were maintained in AIMS aquaria supplied with flowing coastal seawater prior to their incubation in metal-deficient media. Our experiments show that lowering dissolved metal availability increases non-photochemical quenching of photosystem II, and alters intracellular ROS concentrations (Iglic et al. (in prep.); Shick et al. (in review)) and antioxidant enzyme activity (Vogel (in prep.)). The progressive decrease in photochemical efficiency over the duration of the experiment in the high temperature treatments was similar to the chronic photoinhibition observed in other long-term studies of experimental bleaching of corals Warner et al. 1996, 1999; Berkelmans and Van Oppen 2006), a trend that with continued stress can culminate in catastrophic bleaching. This photophysical response was exacerbated by decreasing Fe availability in the amended seawater medium using the terrestrial siderophore Desferrioximine B (DFB), a strong specific Fe (III)-complexing molecule that greatly diminishes Fe availability to phytoplankton (and shown in our work to similarly affect Fe availability to zooxanthellae *in hospite* without other negative consequences to the coral host). This finding shows that iron limitation of corals in natural environments, as implicated indirectly by earlier studies of marine phytoplankton in reef environments (citations), has the potential to increase coral stress under conditions of bright light and high temperature.

Pigment analysis of the test nubbins provided some indication of how cell metabolism was altered by Fe limitation. The was a marked shift in xanthophyll poise toward photoprotection under Fe limitation, partly shunting absorbed photon energy away from Photosystem II (non-photosyntheic quenching - NPQ). As alternatively suggested, however, this shift may stem partly from an additional compensatory defensive role of diatoxanthin as a carotenoid stabilizer of thylakoid membranes as proposed by Dove et al. (2006) or, in view of a lowered concentration of β-carotene in the Fe limited corals, as a supplementary antioxidant to detoxify reactive oxygen species under conditions of chronic photooxidative stress. The absence of nubbin recovery under high light from this shift in xanthophyll composition indicates that photooxidative damage occurred to PSII, exacerbated by decreased Fe availability.

A similar finding was observed in field incubation studies on one of the cruises, whereby the addition of the siderophore DFB to reduce Fe availability resulted in markedly lowered photochemical efficiencies of the endosymbionts in coral colonies from the outer reef environment (Fig. 2). The inner reef is affected annually by the fringes of high riverine and dust inputs, with elevated trace metal concentrations (e.g., dissolved Fe $\geq$ 1 nM), while the outer reef system tends to be more removed (but not free) from this influence. Freshly collected coral colonies were incubated with and without DFB. Colonies were maintained in specially designed chambers on deck under trace metal clean conditions. Colonies collected from Pith Reef, on the outer margin of the Central GBR, markedly decreased their photosynthetic efficiency in the presence of DFB, while corals at Davies Reef, closer to terrestrial inputs of metals, showed no such effect (Fig. 2). Repeating this experiment under conditions of ambient and deplete Fe availability induced by addition of DFB yielded a similar finding, with the decrease in photosynthetic efficiencies being reversed by subsequent exposure to ambient seawater (Fig. 3).

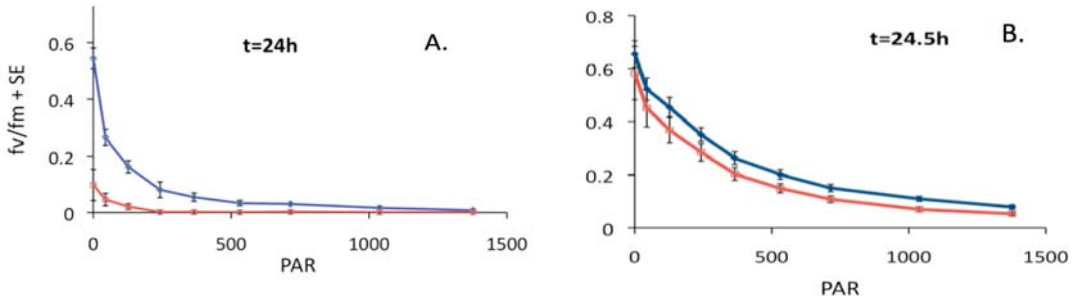

Figure 2. Differences in Photosynthetic efficiency (Fv/Fm) as a function of light intensity for outer (Pith Reef: A) and inner (Davies Reef: B) reef corals after 24 h on-deck incubation. The blue lines show the controls and the red lines treatments with DFB added to reduce Fe availability. These findings show 1) outer reef corals were more susceptible to high-irradiance stress (lower Fv/Fm), 2) decreases in Fe availability exacerbated this sensitivity, and 3) this effect was not seen with inner reef corals that are naturally exposed to higher dissolved Fe concentrations (Wells et al. (in prep.)). The loss of efficiency was not due to decreased chlorophyll but to deactivation of PS II by non-photosynthetic quenching, demonstrated by a rapid recovery of efficiency after Fe addition (not shown)

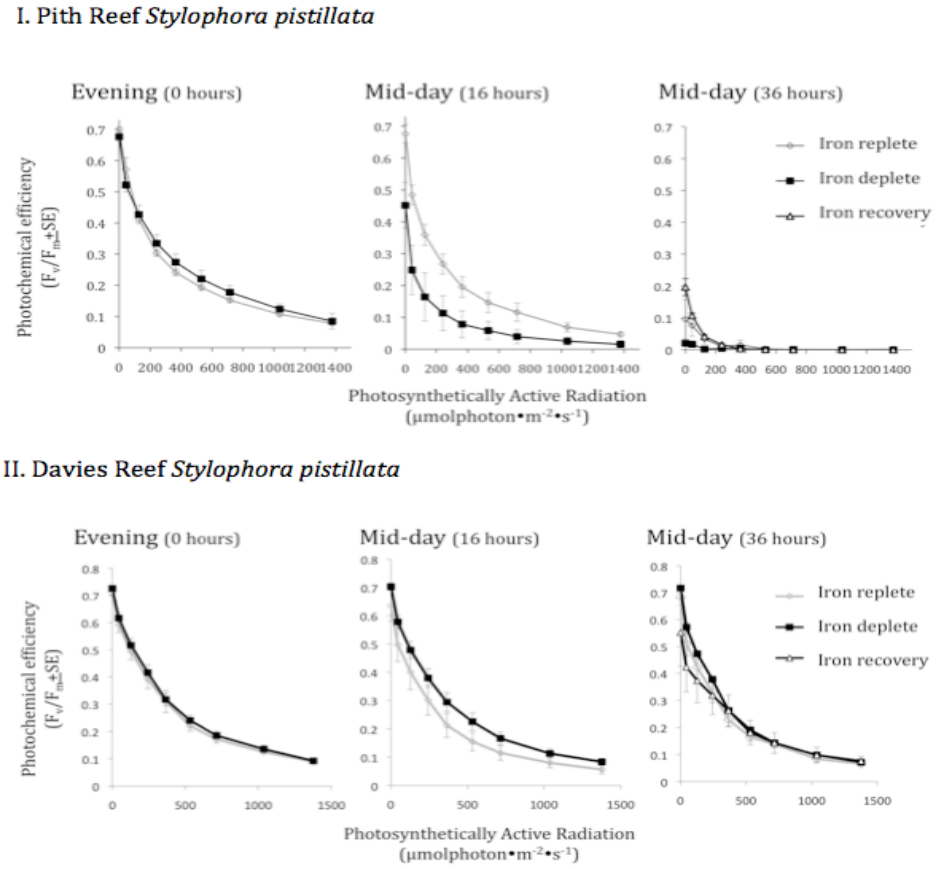

Figure 3. Photochemical efficiency [F_v/F_m±SE] of endosymbionts of Pith Reef (I) and Davies Reef (I) Stylophora pistillata colonies exposed to ambient iron (un-amended seawater) and iron deplete seawater (generated by adding 300 nM of the iron chelator DFB to seawater) conditions. Coral colonies were placed in 2.2 L dust free Sistema plastic chambers with their appropriate

treatments and were ballasted in nally bins on the ship's deck. Each chamber contained one colony and F_v/F_m measurements were taken on three branches of the Pith Reef colony and four branches of the Davies Reef colony to generate the mean and SE. After 16 hours of treatment, the iron deplete coral was divided and half the colony as placed into seawater devoid of DFB, to establish a recovery treatment. Induction curve analysis was conducted on the corals after a 20 minute dark acclimation period to determine the ability of the endosymbiont's to handle acute light stress

Nevertheless, our laboratory experiments show that corals maintained in media enriched with Fe resulted in increased intracellular production of reactive oxygen species within the endosymbionts. That is, augmenting corals with nutritional levels of Fe that are beneficial to marine phytoplankton results in a negative impact when corals are exposed to elevated light and temperature conditions. The findings suggest that Fe may have a pivotal role in the health of coral reef environments, with negative impacts occurring when Fe availability either is very high or very low when high temperatures occur.

Despite these clear findings, it has been difficult to find uniform results on metal limitation effects among experiments done at different times. While individual experiments show good reproducibility among replicates, colonies from different sites, or maintained in the AIMS flowing seawater facility for different amounts of time, show different magnitudes of response. In some cases corals show no discernable effects of metal manipulations. One of the more likely explanations for this outcome is the variance induced by necessarily maintaining the corals in coastal seawater before initiating the experiment. Results show a high degree of variability in trace metal concentrations in these waters that would introduce differences in the endosymbiotic algae's intracellular metal reserves before the experiments began. This finding highlights the importance of establishing a uniform trace metal regime that is lacking in all current laboratory-based coral studies.

Trace Metal Distributions across the GBR: Our findings show that dissolved iron and copper concentrations decrease by an order of magnitude across the coastal to outer reef environment and into the Coral Sea. Not surprisingly, metal concentrations are highest at the coast in both summer and fall, with the highest levels occurring during summer associated with high runoff. There is little variance in metal concentrations with depth on the reef and channel flats, showing little evidence for a sedimentary source of iron and copper, and these values are intermediate with that in coastal and offshore. Surface metal concentrations at the outer region of the shelf break can decrease to very low concentrations typical of oceanic environments, with the oceanographically consistent trend of increasing concentrations with depth. Even here, however, we observed significant variations in surface and deep-water concentrations among seasons and years, implying that nearshore processes can strongly influence the metal nutritional status of even outer reef environments.

These findings suggest that the time-integrated exposure of corals to fluctuating trace metal concentrations may well influence the ability of corals to sustain themselves under conditions promoting oxidative stress. Pelagic phytoplankton are capable of excess, or "luxury", metal uptake under metal-replete conditions, and it is likely that *Symbiodinium* spp. retain this ability *in hospite*. Given our findings of the potential effects of Fe and Cu

on coral oxidative stress, the picture that emerges is one where seasonal and annual variability in trace metal conditions may be a contributing factor to sporadic coral bleaching events in this region. For example, the experimental findings shown in Figure 2 were obtained during a low runoff period. Addition of DFB had no measurable effects on coral photophysiology during the subsequent cruise that followed the summer rainy season, where very large volumes of runoff had influenced the inner and outer reef regions. In other words, during periods of high temperature and light, coral bleaching may be less severe if zooxanthellae have (previously) accumulated sufficient excess metal reserves to facilitate rapid production of metal-based antioxidant enzymes, the fundamental component of our central hypothesis. Alternatively, if Fe is highly enriched (e.g. from dust or other sources), then intracellular Fe concentrations would also be high, potentially increasing the susceptibility of corals to catastrophic metal-induced oxidative stress under elevated temperature and light conditions.

Coral Maintenance under Defined Metal Conditions: We attempted several times to use synthetic seawater in our experiments with corals so that metal limitation could be strictly regulated. We chose the well-known medium AQUIL, used successfully in trace metal studies with phytoplankton, as our base solution. However, despite multiple attempts with different formulations of metals, coral survival was limited to only several days, after which death was rapid. Either a key constituent was missing in this formulation or some trace element or other constituent was toxic. We therefore switched to using natural filtered seawater that was UV-oxidized and run through a chelex resin to remove transition metals, and added these back afterward in metal-EDTA mixes, as done for AQUIL. This approach dramatically improved coral health and enabled us to run more fine-tuned metal limitation experiments, both on shore and at sea.

Effect of Iron Limitation on Reactive Oxygen Species in Coral Zoothanthellae: We used flow cytometry and intracellular fluorescent probes to assess the photophysiology of freshly isolated endosymbionts from *Stylophora pistillata*. Intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) were measured using the fluorescent dyes dihydroethidium and CMH_2DCFDA, and cell membrane integrity was assessed using SYTOX, a cell-wall impermeable dye. We hypothesized that greater photophysiological stress would occur in endosymbionts from *S. pistillata* colonies kept at high temperature (31°C) relative to control colonies (27°C). Also, stress might be greater still at higher temperatures when iron availability was low, conditions created by adding the strong iron chelator desferrioxamine B to seawater bathing the colonies. Our findings that endosymbionts contained a significantly *lower* level of ROS (superoxide radical) and maintained a *higher* than predicted level of cell membrane integrity under low iron, high temperature conditions are suggestive of an induced (photo)protective mechanism. These results are consistent with changes in photoprotective xanthophyll cycling (diatoxanthin:diadinoxanthin ratio) measured in these endosymbionts (see below). The results imply that diminished iron availability renders corals more likely to decrease their photosynthetic efficiencies under pre-bleaching conditions, but that the gradual implementation of temperature stress used in our experiments provided the zoothanthellae time to increase non-photochemical quenching of PSII and thereby avoid oxidative stress.

We performed an irradiance experiment under ambient (field) metal conditions, where corals were maintained under increasing levels of natural PAR in outdoor aquaria. The findings show photosynthetic efficiencies decreased progressively with increasing PAR, and these changes are correlated with increasing intracellular fluxes of ROS. This "control" experiment confirms that ROS production in zooxanthellae provides a sensitive measure of photophysical stress, and reinforces the interpretation of our findings above.

Two short-term Fe limitation experiments were performed to compare the Fe limitation response in terms of ROS production in endosymbionts of *S. pistillata* and *Acropora millepora*. While there were no appreciable differences seen in ROS production between the algae in colonies of these two species that had been maintained at AIMS (but note above), there were clear differences between the two species found in their Fv/ Fm response to light. These findings suggest that the endosymbiont photosystems are responding to light and temperature far faster than their antioxidant enzyme systems. One indication is that *A. millepora* is more influenced by Fe stress than is *S. pistillata*, although more testing would be necessary to confirm this.

In summary, the combined effect of temperature stress and iron limitation on the endosymbionts of *S. pistillata* is multifaceted. Temperature stress alone caused a decrease in freshly isolated endosymbiont PSII photochemical efficiency and intracellular ROS. The enhanced negative effect of iron limitation on Fv/Fm at high temperature may be attributable to decreases in Fe-rich photosynthetic electron transport chain components or increased xanthophyll cycling, both of which suggestions are supported by the decrease in intracellular ROS and RNS measurements in freshly isolated endosymbionts exposed to iron limitation and high temperature within this study. This study suggests that iron limitation and temperature stress generate protective responses in endosymbionts of *S. pistillata*, manifested by decreases in ROS and RNS and increases in xanthophyll pigment cycling.

Effect of Metal Limitation on Antioxidant Enzyme Activity of the Coral Host and Endosymbionts: Measuring antioxidant enzyme activities in the very small sample sizes available with our multivariate coral experiments essentially was not possible with the standard protocols used prior to this study. We modified, tested and verified a new low volume enzyme analysis protocol that employs well-plate readers thereby increasing sensitivity dramatically and allowing for more accurate assessment of enzyme activities in our metal manipulation experiments. We routinely analyzed the activities of Mn, Fe, and Cu/Zn superoxide dismutases (by preparing standard curves using commercial standards of these isoforms of SOD), catalase, and ascorbic peroxidase in both the coral host and endosymbionts. In most cases, enzyme activities were measured at the beginning and end of the coral incubation experiments, though in some cases mid-point samples also were analyzed.

The findings show that antioxidant enzyme activities are a sensitive indicator of temperature and light stress in corals, but these activities have an inconsistent response to changes Fe, Cu, Zn and Mn availability. While activity of all three forms of SOD (Mn, Cu/Zn, and Fe SOD) were measured in some *S. pistillata* colonies, MnSOD dominated the antioxidant pool in all samples, with Cu/Zn SOD's being less prevalent and FeSOD

being comparatively rare. In some experiments there were clear indications of metabolic substitution of antioxidant enzyme activities, whereby one metal-containing enzyme was substituted for another under some metal limitation conditions. For example, decreasing Cu and Zn availability results in strong increases in MnSOD and (Fe containing) ascorbate peroxidase activities under high light conditions in both the host and endosymbionts, presumably resulting in continued control of ROS. Corals and endosymbionts under limiting Mn conditions show no MnSOD activities under high light, and there is no recovery of MnSOD activity over the short term by augmenting the media with Mn. Even so, subsequent experiments with colonies having different legacy exposure to trace metal regimes generated different results. While our data analysis and interpretations continue at this point, the findings suggest that the complex metabolic response of corals and their endosymbionts to high temperature and light, combined with the likely influence of legacy exposure to varying nutrient metal availability, makes it difficult to ascertain with high confidence the impact seawater metal conditions have on the ability of corals to circumvent ROS production, and the effectiveness of their antioxidant defenses.

Effect of Iron Limitation on Pigment Composition of Zoothanthellae: Decreased photosynthetic efficiencies in pelagic phytoplankton can be related to iron limitation, but this effect had not been tested in corals. We found that reducing iron availability to *Stylophora pistillata* colonies at 27°C (by adding desferrioximine B) yields decreased cellular concentrations of photosynthetic pigments (chlorophyll *a*, c_2, peridinin-protein complexes) in their FIZ. Iron-replete colonies maintained under moderate heat stress (31°C) yield similar pigment concentrations with or without iron limitation. Decreased iron availability also affected diatoxanthin (DT)–diadinoxanthin (DD) balance, a photo-protective mechanism that limits over-reduction of PSII by increasing non-photochemical quenching. Under higher temperature and reduced iron availability, the DT/(DD + DT) ratio in freshly isolated endosymbionts increased 3-fold relative to lower temperature conditions, regardless of the level of available iron, corresponding to decreased Fv/Fm *in hospite*.

Combined, these findings show that 1) decreasing iron availability in seawater affects the photophysiology of zoothanthellae *in hospite*, and that this effect is greatest under higher (stressful) temperatures, 2) the decrease in photosynthetic efficiency under these conditions results from an increase in non-photochemical quenching by increasing photoprotective pigments, and 3) that these steps are sufficient to lower the production of ROS when temperature increases are slow enough to provide corals and their zoothanthellae time to adjust or acclimate to the more stressful conditions. The findings clearly show that decreases in iron availability in seawater could exacerbate the declining photosynthetic efficiencies in *Stylophora* colonies under elevated temperatures in nature.

Comparison of the Responses of Inner- and Outer-Reef Adapted Corals to High Temperature and Light Under Metal-Replete and -Deplete Conditions: Given the evidence that "legacy" exposure of corals to different levels of nutritional trace metals may influence their response to high light and temperature (see above), we measured the effects of trace metal limitation on two geographically separated colonies of the same species, *S. pistillata*: a coastal colony collected from Long Island in the Whitsunday

Islands, and an outer shelf colony from Pith Reef. Briefly, colonies from each location were maintained in basal natural seawater stripped of trace metals (UV oxidation followed by Chelex™ ion exchange) with the nutrient metals added back (or not – Fe) at optimal concentrations for growing phytoplankton. The findings showed significant changes in photosynthetic efficiencies at elevated temperature, with greater decreases on the final day under Fe limitation in the Pith Reef colony than with the Whitsunday colony, consistent with our earlier findings. However, while the Whitsunday isolates showed some decrease in dark-adapted Fv/Fm under conditions of Fe limitation, a similar decrease was observed with full trace metal supplementation at high temperature. That is, supplementing the media with other bioactive metals (Mn, Zn, Cu) at slightly elevated concentrations over natural levels had the same negative effect on overall photosynthetic efficiency as Fe limitation. The decline in dark-adapted photochemical efficiency (F_v/F_m) at high temperature was coincident with increases in detected intracellular ROS and RNS and decreased membrane integrity, suggesting that the decrease in the efficiency of charge separation within PSII (Fv/Fm) was one of the main source of oxidative stress in the algal symbionts. The cause for this effect is not clear, but the implication is that natural fluctuations in metal concentrations have the potential to induce significant oxidative stress responses in corals.

Overall, this study illustrates an enhanced negative effect of Fe limitation on the thermally-depressed PSII photochemical efficiency of symbiotic dinoflagellates of *S. pistillata* isolated from two distinct geographical areas. Furthermore, this negative effect of high temperature on photochemistry was manifested as an increase in intracellular oxidative load; however, Fe limitation did not have the same effect on colonies of *S. pistillata* isolated from different locations, potentially reflecting a differential response to long-term natural trace metal availability at the two sites.

Effect of Fe Limitation on Oxidative Stress in Cultures of *Symbiodinium*: We assessed the relationship between Fe limitation and oxidative load within two strains of symbiotic dinoflagellates maintained in culture when exposed to elevated temperature and irradiance. The enhancement of oxidative load, shown by elevated fluorescence of intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) probes, within both *Symbiodinium* strains under Fe limitation and elevated temperature (predominantly but not exclusively Strain 2432) and irradiance (predominantly but not exclusively Strain 831) supports a causal link between Fe limitation and oxidative stress. The findings suggest the response was due to the impairment of the photosynthetic electron transport chain or antioxidant enzyme activity resulting from Fe's critical role in the structure and function of photosystems and antioxidant enzymes. As found in our preliminary studies, the differences between ROS and RNS load of each *Symbiodinium* strain suggests that these (and likely other strains) differ in their susceptibility to trace metal limitation, particularly under thermal and irradiance stress. The findings indicate that Fe limitation of *Symbiodinium in hospite* may cause elevated levels of ROS and RNS under temperature and irradiance stress, stimulating coral bleaching events, according to the oxidative theory of coral bleaching. These laboratory findings directly support the foundation of our interpretation of the coral colony studies.

Genetic Assessment of *Stylophora pistillata* across the Central GBR Region: As previously mentioned, obtaining uniform, reproducible results on metal limitation effects among our experiments was challenging. Whether this was due to seasonal changes, differences in local concentrations of trace metals, or differences on a population level was not clear. To address this problem we conducted a phylogenetic assessment of *S. pistillata* on a small geographical scale across the GBR from the inner to outer reef sites, representative of our field scale sampling for dissolved Fe and Cu on the GBR. Mitochondrial and nuclear markers revealed no significant levels of genetic variability within the geographical distribution of this species considered here. Minor genetic differences were encountered and these do not always correspond with geographically defined populations. This is indicative of a genetically homogeneous species and high levels of gene flow among populations. The exception are colonies from the offshore Flinders Reef system in the Coral Sea, where nuclear markers showed several synapomorphies, indicating that this population is genetically isolated from the rest that we tested. Given the important role of *S. pistillata* as a model species for physiological studies, we consider the encountered genetic homogeneity in the studied geographical scale to be a very important finding. More specifically, these findings confirm that we can properly assess the effects of metals on oxidative stress in the model species we have chosen without major concern about genetic differences underpinning different physiological responses. Results also indicate there are differences between *S. pistillata* that we are studying on the GBR and those collected in the Red Sea and China Sea. This finding may have implications for comparing our findings to studies that assess oxidative stress responses of *S. pistillata* in other regions.

In summary, the assessment of genetic diversity in our experimental populations of this species was successful. We detected some minor local variation that is generally acceptable within a single morpho-species and considering the distances in the reef and the myriads of microhabitat for selection. Hence, we consider *S. pistillata* collected in our study suitable for the assessment of functional gene expression using a GeXP (Beckman Coulter (Fullerton, CA, USA) GenomeLab_GeXP Genetic Analysis System) assay. The findings of the diversity study of *S. pistillata* were presented at the Euro ISRS symposium, Wageningen, Netherlands in December 2010 and a manuscript will be submitted shortly to the journal *Diversity and Distribution*.

Differential Gene Expression: This component of the project aimed to identify changes in functional gene expression related to trace metal limitation for the coral host as well as the~~ir~~ symbiotic algae. The overall goal was to better understand the transcriptional response in reef-building corals to changing environmental conditions conducive to coral bleaching. We have performed multi-locus PCR based assays of gene expression specifically designed for *S. pistillata* to better assess oxidative stress indicators in response to the varying trace metal conditions. We identified appropriate target genes, designed primers and optimised the assay before applying it to our experimental samples. We composed a precise multiplex reverse transcription quantitative polymerase chain reaction (RT–qPCR) assay, capable of reproducing gene expression profiles from 18 target genes in *S. pistillata*. The 12 gene of interest have known or putative roles in the coral's cellular response to oxidative stress. We have applied the gene assay to samples collected across our field scale (see above) as well as to samples exposed metal limitation

treatments. At this point we have data for three papers that we anticipate submitting by the end of this year.

The Proteome of Symbiotic Dinoflagellates Isolated from Colonies of *S. pistillata* Subjected to Trace-Metal Limitation and High Temperature: Although differential gene expression assessed by transcriptomic techniques has provided valuable information regarding coral responses to environmental stress at the molecular level, the transcriptome does not represent the full phenotype of the coral symbiosis. It has been estimated that only 30% of a cell's phenotype can be described by the transcriptome compared to 80% by the proteome (Feder and Walser 2005). Only a few studies have examined the proteome of symbiotic or aposymbiotic cnidarians using two-dimensional SDS-PAGE analysis. The success of such attempts was restricted by the applicability of low-throughput protein analyses. Therefore we used high-throughput protein analysis by LC-MS/MS and Tandem Mass Tag (TMT) reagents to obtain the first comprehensive proteome expression profile of endosymbiont-enriched fractions taken from an intact coral colony to examine the response of the endosymbiont to several conditions of temperature and trace-metal limitation. TMT tagging results in the production of reporter ions that allow quantification of tagged peptides; untagged peptides are identifiable but not quantifiable.

Of the 109 peptides meeting the stringent criteria for quantification, 83 (76%) did not show any significant change in TMT tag intensity compared to the unstressed proteome at t=0 prior to the application of thermal stress or trace-metal restriction. Of the remaining 26 assigned peptides where differences in TMT tag intensities between treatments relative to the pre-treatment t=0 proteome were found to be statistically significant, 14/26 peptides have a significant reduction in TMT tag intensity compared to the unstressed t=0 proteome in at least one of the low temperature treatments. Only 6/26 assigned peptides showed a statistically significant increase in TMT tag intensity compared to the unstressed t=0 proteome; these were all associated with high temperature treatments. Overall, it appeared not to be metal limitation *per se* that influenced TMT tag intensity, but rather high temperature, irrespective of the presence of Fe. That is, at the higher temperature, significant increases in TMT tag intensities occurred in five proteins in both HT –Fe and HT +Metal conditions, and an additional four proteins showed significant increases only in HT +Metal, and a further three proteins only in HT –Fe. Corals in the HT –Mn treatment perished before symbiotic algae could be harvested.

Presence of the common antioxidant enzymes in the quantitative data was expected. Surprisingly, however, only nitric oxide reductase and a probable thiol peroxidase could be quantified, with the TMT tag intensity of the nitric oxide reductase being significantly lower in the low temperature treatments. The intensity of the thiol peroxidase TMT tag did not change significantly compared to the pre-treatment t=0 proteome in any experimental condition. Interestingly, this protein uses an iron co-factor, as does nitric oxide reductase, but the TMT tag intensity in the high temperature treatment with iron limitation did not change significantly compared to the unstressed t=0 proteome. Only two proteins associated with apoptosis or exocytosis (two mechanisms whereby algal endosymbionts may leave host cells during coral bleaching) were quantifiable, and

neither changed significantly with experimental conditions. Given that TMT-tagging is proportional to protein concentration and that NOR was tagged at high yield, this protein may be produced by the algal partner to protect against the damaging effects of the nitric oxide (NO) generated by the host, where NO has been implicated in the initiation of the bleaching response.

There were an additional 8098 unique proteins that could be separated to allow sufficient amino-acid sequence to be used to search Uniprot. Of those 8098 proteins, 7790 proteins could be further assigned a biochemical function based on KEGG gene ontology descriptors. These included the antioxidant enzymes of interest (SODs, catalase, and ascorbate peroxidase), but these were not labeled and therefore were unquantifiable.

Among the surprises afforded by the proteins in the qualitative database was the presence of a diversity of toxins known from various bacteria, fungi, invertebrates, and vertebrates. None of these toxins were encoded in the recently reported genomes of corals in the genus *Acropora*. Thus, despite our initial assumption that the toxins in our proteome of the dinoflagellate symbionts of *Stylophora* were from contamination of the algal isolates by fragments of host coral nematocysts, this seems not to be the case, and the toxins (not previously reported in free-living dinoflagellates) may instead be associated either with the endosymbionts or perhaps with unspecified bacterial associates.

Photosystem proteins were present as expected for *Symbiodinium*, but the unsuspected presence of phycoerythrin and phycocyanin gives evidence that our corals harbored cyanobacteria as either consorts or pathogens. Likewise, present in the qualitative database were proteins associated with nitrogen fixation, which is not known in algal endosymbionts such as *Symbiodinium*, and these proteins could not be found encoded in coral genomes, suggesting either that *Symbiodinium* can fix dissolved nitrogen or more likely these nitrogenases are from coral-associated cyanobacteria or from diazotrophic bacteria (predominantly γ-proteobacteria) that in coral are strongly correlated with dinoflagellate abundance, suggesting a tight physiological relationship between these heterotrophic and phototrophic partners.

The presence in the endosymbiotic algae of urease and uricase may indicate a role of these enzymes in liberating nitrogen found in these waste products from the host. Additional enzymes of inorganic nitrogen assimilation (glutamine and glutamate synthetases) also are present.

We also found several members of the highly conserved heme superfamily of proteins to be abundant in the endosymbionts. These hemoproteins may function as post-translationally activated redox-regulated nitrite reductases that could mediate the NO response to cause cnidarian bleaching. Separate from the hemoproteins of metabolism and photosynthesis, these oxygen-binding globins may serve additionally as O_2-sensors to activate cellular defence pathways by regulating the transcription of protective oxygen-responsive genes, particularly those factors activated in response to hypoxic conditions that prevail in respiring coral tissues in the dark. Expressed also are the ubiquitous ferritin intracellular Fe-storage proteins of heme metabolism, which are reported to be

transcriptionally up-regulated by corals in response to thermal stress, perhaps as a means of keeping concentrations of free intracellular Fe low to avoid its toxic effects.

Returning to the quantitative dataset, a remarkable finding was the 114-fold increase in viral replication protein under metal-replete conditions at high temperature. Examination of the qualitative data for viral proteins indicates the complete range of proteins required for viral replication, production of new viral particles and viral – host interaction, especially RNA-dependent RNA polymerase, which would indicate the presence of negative-strand RNA viruses. It would seem from these proteomics results that virulence is high during coral stress. Viral replication requires new viral particles to be transmitted to new host cells, these processes include viral budding or host cell lysis in either the infected coral tissue or its endosymbionts. Large-scale perturbation of the host cell membrane or host cell lysis would indicate that corals under stress would exhibit a high level of tissue necrosis or a symbiont that has been associated with severe coral bleaching in the past. We suggest that coral recovery post-bleaching not only depends on the acquisition (or retention) of heat tolerant endosymbionts but also on reducing the viral burden of the coral to pre-bleaching levels. Although viruses have been implicated as a cause of coral disease and bleaching, the complexities of coral-viral interactions are poorly understood, particularly in connection with environmental change, and clearly warrant future investigation.

Opportunities for Training and Development:

The project has supported the research and training efforts of three graduate students: Kathryn Vogel (U. Maine) working with co-PIs Malcolm Shick and Mark Wells, and Katrina Iglic, a student just completing her PhD with co-PI Charles Trick at the University of Western Ontario. In addition, the project supported the summer participation of Victoria Hewlett, a junior M.Sc. student who assisted with the coral experiments. The project supported the part-time involvement of an undergraduate student, Sonja Allen, who graduated in 2011 from U. Maine with degrees in Marine Science and Studio Art, and who did many of the measurement of coral surface area using 3-dimensional laser scanning. Over the last year the project also supported Dr. Anke Klüter as a Postdoctoral Scholar, who has been conducting the gene expression studies described here.

Outreach Activities:

We have worked with media outreach at U. Maine to develop both media stories for the official U. Maine web portal and local newspapers, as well as a photo-rich story in the U. Maine Alumni magazine. Talks have been given on corals and bleaching at local schools. The work also was featured in an Australian (ABC) radio interview with co-PIs Dunlap, Wells, and Shick conducted at AIMS. Published results to date were featured in NSF's *Science360* (http://news.science360.gov/archives/20110829).

Contributions to Principal Discipline:

The project focuses on the primary mechanisms underlying the coral bleaching response to elevated temperature. This bleaching phenomenon is reasonably well characterized in many specific situations, but is not fundamentally understood, apart from a general consensus that oxidative stress is involved in the loss of algae from the coral host. Elevated temperature, in the presence of light, is needed to cause coral bleaching, but elevated temperature and light conditions are not always sufficient to cause bleaching in natural environments; other unidentified factors appear to be involved. By seeking the fundamental physiological basis for coral bleaching we will be able to assess why conditions conducive to bleaching in natural environments do not always result in bleaching.

Contributions to other disciplines of science:

Coral reefs are vital reservoirs of marine diversity, supporting not only reef dwelling organisms but also broader reef-associated ecosystems, including some higher trophic levels in pelagic environments. Ascertaining the specific physiological factors leading to, or exacerbating, coral bleaching not only provides a baseline understanding for how and why this ecosystem may change in the future, but also potentially illustrates novel mitigation approaches to reduce or minimize future trends in coral bleaching.

The Development of Human Resources:

The project directly supported one full-time graduate student and one undergraduate working part-time on the project at U. Maine, as well as indirectly, in the form of research opportunities, two full-time graduate students at the University of Western Ontario. All four are female students. A female Postdoctoral Scholar working on the project at AIMS was supported by the project, which also provided part time support for a female technician at U. Maine.

Physical, Institutional or Information Resources:

Nothing to report at this time.

Other aspects of Public welfare beyond science and engineering:

The work here may lead to practical methods for reducing or remediating coral bleaching events.

Publications, Presentations and Papers in Preparation:

Shick, J.M. 2008. Toward an aesthetic marine biology. ***Art Journal***, 67 (4), Winter 2008: 62–86. (This paper incorporates examples of "coral biology," including bleaching, in fine and decorative arts used in teaching undergraduate marine science majors and non-science Honors students.)

Shick, J.M., Iglic, K., Wells, M.L., Trick, C.G., Doyle, J., Dunlap, W.C. 2011. Effects of iron availability on the response of the coral *Stylophora pistillata* to light and high temperature: Implications of trace-metal limitation for coral bleaching. ***Limnology and Oceanography*** 56:813-828

Klüter, A., Andreakis, N. 2010. "Phylogenetic assessment of the scleractinian coral *Stylophora pistillata* on a small geographical scale". European ISRS Symposium, Wageningen, Netherlands

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Shick, J. M., Iglic, K., Wells, M. L., Trick, C. G., Dunlap, W. C. 2009. Iron limitation reduces photosynthetic efficiency in *Stylophora pistillata* colonies at high temperature. ASLO Winter Meeting, Nice, France.

Trick, C. G., Shick, J. M., Wells, M. L., Doyle, J., Iglic, K., Hewlett, V. B., Dunlap, W. C. 2009. Iron and temperature effects on photosynthetic and photoprotective pigments in *Stylophora pistillata* zooxanthellae. ASLO Winter Meeting, Nice, France.

Iglic, K., C.G. Trick, W.L. Wells, M. Shick, and W. Dunlap (in preparation) Flow cytometric measurements of oxidative stress in freshly isolated algal symbionts of corals as a function of temperature and iron availability (*Limnology and Oceanography*).

Iglic, K., C.G. Trick, W.L. Wells, M. Shick, A. Klüter, and W. Dunlap (in preparation) Photochemical and oxidative responses of the symbiotic dinoflagellates of *Stylophora pistillata* colonies from Pith Reef and Long Island of the Whitsunday Islands of the Great Barrier Reef. (*Coral Reefs*).

Iglic, K., C.G. Trick, W.L. Wells, M. Shick, and W. Dunlap (in preparation) The stimulation of oxidative stress in temperature stressed, iron depleted cultures of *Symbiodinium* grown under continuous culture conditions. (Limnology and Oceanography).

Klüter, A. and Andreakis, N (in preparation) Assessing genetic diversity in the scleractinian coral *Stylophora pistillata* (Esper 1797) from the Central Great Barrier Reef and the Coral Sea. (*Diversity and Distribution*)

Klüter, A., N. Andreakis, W.C. Dunlap, J.M. Shick, M.L. Wells (in preparation) Multi-locus PCR based gene expression assay in *Stylophora pistillata*: Implications for management and conservation. (*Journal of Biological Research*)

Klüter A., J. Doyle, B. Leggat, W.C. Dunlap, J.M. Shick, M.L. Wells (in preparation) Field study: inshore to off-shore (Gene expression, Zx genotyping, PAM, pigments). (*Comparative Biochemistry and Physiology A*)

Klüter,A., N. Andreakis, K. Iglic, K. Vogel, J. Doyle, W.C. Dunlap, J.M. Shick, M.L. Wells (in preparation) CuZn short-term and Mn short- and long-term experiments (Gene expression, Enzyme, PAM, Pigments. (*Molecular Ecology*)

Vogel, K., J.M. Shick, M.L. Wells, W.C. Dunlap, A. Kluter, A. and K. Iglic (in preparation). Trace metal effects on antioxidant enzyme activities in *Stylophora pistillata*

colonies and their dinoflagellate endosymbionts from the Great Barrier Reef. (*Coral Reefs* or *Biological Bulletin, Woods Hole*)

Wells, M.L., C.G. Trick, J.M. Shick, and W.C. Dunlap (in preparation). Dissolved iron and copper distributions across the inner and outer Great Barrier Reef. (*Marine Chemistry*)

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Shcolnick, S., and N. Keren. 2006. Metal homeostasis in cyanobacteria and chloroplasts. Balancing benefits and risks to the photosynthetic apparatus. Plant Physiol. **141:** 805-810.

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Vogel, K. (in prep.). Superoxide dismutase, ascorbate peroxidase and catalase activities in symbiotic corals and zooxanthellae under trace metal limitation. University of Maine.

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