Analysis of Biophysical, Optical and Genetic Diversity of DoD Coral Reef Communities Using Advanced Fluorescence and Molecular Biology Techniques

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LIST OF ACRONYMS

AOV Autonomously Operated Vehicle
ATP Adenosine 5'-triphosphate
CW Continuous Wave
GFP Green Fluorescent Protein
FIRe Fluorescence Induction and Relaxation technique
FRRF Fast Repetition Rate Fluorometry
HPLC High-Performance Liquid Chromatography
ITS I Internal Transcribed Spacer region
LED Light Emitting Diode
PCD Programmed Cell Death
PCR polymerase chain reaction
PQ Plastoquinone
PSI Photosystem I
PSII Photosystem II
ROS Reactive Oxygen Species
ROV Remotely Operated Vehicle
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

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1. EXECUTIVE SUMMARY

DoD has responsibility for numerous facilities in tropical and subtropical environments that are adjacent to coral reefs. Coral reefs are specifically susceptible to anthropogenic insult and are rapidly degrading worldwide. Documenting the physiological state of reef communities is critical for developing remediation strategies that can both reduce anthropogenic impact and distinguish between natural stress and anthropogenic factors potentially related to military activity. The ability to characterize, assess, and monitor underwater benthic communities associated with DoD sites or activities is required in order to document compliance with promulgated national policy and to ensure that DoD operations do not lead to natural resource degradation, particularly with respect to coral reefs. Executive Order 13089 "Protection of Coral Reefs" dated June 11, 1998 directs Federal agencies including DoD to study, restore, and conserve U.S. coral reefs. Specifically, E.O. 13089 directs Federal agencies whose actions may affect U.S. coral reef ecosystems, to take the following steps: (1) Identify actions that may affect U.S. coral reef ecosystems (2) utilize programs and authorities to protect and enhance the conditions of such ecosystems, and (3) to the extent permitted by law, ensure that any actions they authorize, fund, or carry out, will not degrade the conditions of such ecosystems.

This R&D project was designed to serve the basic objectives of SON SISON-03-02 "Assessment of Benthic Communities for the Department of Defense" with the overarching goal to provide quantitative baseline data, as well as advanced methods and ready-for-transition technology needed for the assessment of benthic ecosystems at DoD installations. The specific objectives of this project were to develop advanced techniques for non-destructive assessment of the viability and health of coral reef communities with the capabilities of identification and quantification of natural and anthropogenic stresses, design "bench scale" prototypes of Fluorescence Induction and Relaxation (FIRe) Fluorosensors for permanent underwater monitoring stations and Remotely Operated Vehicles, and to collect baseline data on physiological, biophysical, bio-optical and genetic diversity of coral reef communities in the Caribbean and the Indo-Pacific regions.

The novel Fluorescence Induction and Relaxation (FIRe) technique and instrumentation have been developed for measuring photosynthetic characteristics and assessing the physiological status of coral and other benthic organisms (Gorbunov and Falkowski, 2004). This advanced technique relies on the established relationship between fluorescence emission and the efficiency of photosynthetic processes and provides a comprehensive suite of fluorescent and photosynthetic parameters of the target. The measured parameters reflect the physiological status of various components of the photosynthetic machinery, including the primary photochemical reaction in Photosystem II reaction centers and the electron transport within and down to Photosystem II (i.e., secondary photosynthetic reactions). These photosynthetic processes and related fluorescent parameters are particularly sensitive to environmental stresses, such as nutrient availability, irradiance, temperature, and anthropogenic insults, thus providing a biophysical background for non-invasive fluorescence monitoring of the organisms. The research revealed that the FIRe parameters are very sensitive to changes in the coral physiology and alert detrimental changes at early stages of the stress development - before any visible changes in coral coloration appear.

A bench-top version of the FIRe System has been developed and constructed and employed in the lab and field research. On the second stage of the instrument development, an underwater multi-receiver FIRe System for long-term underwater monitoring stations has been designed and tested. This system permits simultaneous monitoring *in situ* of multiple targets, a crucial factor in sampling extraordinary diverse coral reef communities. Finally, a bench-scale prototype of laser-based FIRe Sensor has been designed for Autonomous Underwater Vehicles (AOV). Based on the experiments in coral tanks, the specifications for a future operational ROV/AOV FIRe sensor have been defined.

To conduct controlled manipulation experiments on live coral in the lab, Coral Cultivation Facilities were built at Rutgers University. The biophysical and biochemical parts of the project were completed both in the lab and in field with the aim to elucidate the impact of common natural stresses (as elevated temperature and excess light) and selected anthropogenic stresses (such as heavy metal contamination) on coral. The cellular and molecular mechanisms, together with the optical signatures of the stresses have been established (Tchernov et al, 2004). On this background, bio-optical algorithms for detection and assessment of the stresses have been developed and evaluated. During the field studies in the Caribbean and Pacific regions, the baseline information on the variability in fluorescent and photosynthetic properties of corals have been collected.

Due to the presence of unique pigments (fluorescent proteins) in cnidarian hosts, corals are characterized by the amazing variety of colors. The diversity of the color palette is an important indicator of the health and viability of coral ecosystems. The molecular biology and genetic part of the project included identification, isolation, as well as spectroscopic, genetic and biochemical characterization of major color determinants in corals, as well assequencing, cloning, and characterization of 30 new fluorescent proteins from corals. Empirical correlation between individual allelomorphs and the photosynthetic and physiological variability retrieved using the FIRe variable fluorescence techniques were further examined. Although further research is needed to understand the physiological function and extraordinary variability of expression of fluorescent proteins in coral, this part of the project provides scientific background for understanding the optical diversity of coral reef ecosystems and interpreting the results of optical monitoring of benthic communities using spectral fluorescence and reflectance measurements.

The technology transfer of the bench-top version of the FIRe System to a small hi-tech company, Satlantic Inc., has been completed (www.satlantic.com/fire). A diver-operated fluorometer system and a moored FIRe fluorometer system and bio-optical algorithms are proposed to be validated at selected NAVY sites and directly transferred to Naval Facilities Engineering Command Atlantic, via Naval Facilities Engineering Service Center for use at DoD coral reef sites (a pending ESTCP project in partnership with Space and Naval Warfare Systems Center, San Diego).

2. PROJECT BACKGROUND

The DoD is responsible for numerous facilities in tropical and subtropical environments that are adjacent to coral reefs. Coral reefs are specifically susceptible to anthropogenic insult and rapidly degrade worldwide. The development of advanced technologies for environmental monitoring of benthic communities under DoD jurisdiction requires an understanding of how different environmental factors affect the key elements of the ecosystems and the selection of specific monitoring protocols that are most appropriate for the identification and quantification of particular stresses. Documenting the environmental state of reef communities is critical to developing remediation strategies that can both reduce anthropogenic impact and distinguish between natural stress and anthropogenic factors potentially related to military activity. The quantitative assessment of the impact of stresses requires the accurate knowledge of baseline biophysical, optical, and genetic parameters for "healthy" coral reef communities. Due to natural variability within populations and diversity amongst species, these parameters vary both within and between species.

3. PROJECT OBJECTIVES

The objectives of the project were:

- To develop advanced techniques for rapid and non-destructive assessment of the viability and health of coral reef communities with the capabilities of identification and quantification of natural and anthropogenic stresses;
- To develop "bench scale" prototypes of Fluorescence Induction and Relaxation (FIRe) Systems for permanent underwater monitoring stations and Remote Operated Vehicles or Diver Propulsion Vehicles; and
- To collect an extensive library of baseline data on physiological, biophysical, bio-optical and genetic diversity of coral reef communities near DoD installations.

4. TECHNICAL APPROACH

Because photosynthesis is the ultimate source of energy for all shallow water communities, photosynthetic organisms are absolutely critical components in the viability of coral reef ecosystems. Corals are symbiotic associations between an invertebrate host and a photosynthetic alga, called zooxanthellae. Our basic approach for assessment of the health and viability of the photosynthetic organisms is measurement and analysis of chlorophyll variable fluorescence, a property unique to the photosynthetic machinery (Falkowski et al, 2004). The variable fluorescence is recorded by using Fast Repetition Rate (FRR) Fluorometry (Kolber et al, 1998) or its successor, Fluorescence Induction and Relaxation (FIRe) technique (Gorbunov and Falkowski 2004). The optical measurements are sensitive, fast, non-destructive, and can be done in real time and *in situ*. The variable fluorescence technique relies on the relationship between chlorophyll fluorescence and the efficiency of photosynthetic processes and provides a comprehensive suite of fluorescent and photosynthetic parameters of the organism (Kolber et al, 1998; Gorbunov et al. 2000, 2001). Several types of instruments have been used in this research,

including a bench-top FIRe fluorometer (Gorbunov and Falkowski 2004), diver-operated fluorometer (Gorbunov et al. 2000), and moorable FRR fluorometer (Gorbunov et al. 2001). Together with spectral fluorescence and novel molecular biology techniques, the FRR and FIRe fluorometries provide a comprehensive diagnostics of the viability of DoD coral reef ecosystems.

Baseline data on biophysical and optical diversity of coral reef communities has been collected by using our SCUBA-based FRR fluorometers. The following fluorescent and photosynthetic parameters have been recorded and analyzed: the quantum yields of fluorescence at the minimum and maximum levels (F_o and F_m, respectively), the quantum efficiencies of photosynthetic light utilization (F_v/F_m and $\Delta F'/F_m'$), the functional absorption cross section of Photosystem II, the rates of photosynthetic electron transport, and, for selected species, coefficients of photochemical and non-photochemical quenching and parameters of the photosynthesis versus irradiance curve (Kolber et al., 1998; Gorbunov et al. 2001). Based on our previous experience with Caribbean coral reef communities, we have selected from 10 to 20 dominant species in each area for detailed sampling at a variety of depths and irradiance levels. The FRRF signatures are complemented with the spectral and genetic data obtained in the molecular biology part of the project. Although we primarily focus on corals as the key element of coral reef ecosystems, FRRF-derived parameters are also documented for other benthic photosynthetic organisms, including dominant species of macroalgae, seagrasses and epibenthic algal turfs. The collected database of fluorescent and photosynthetic signatures was used as a background for the development and parameterization of the advanced algorithms for optical, non-invasive characterization and discrimination of different functional groups of benthic organisms.

In the first year, a special emphasis was given to studying the impact of natural environmental stresses, such as elevated temperatures and excess irradiance on coral. These are most common natural stresses that may be superimposed to specific anthropogenic stresses related to military activity on DoD installations. The experiments have been conducted both in the laboratory, on isolated cultures of symbiotic zooxanthellae, and in the field, on live corals. For the laboratory experiments, we have constructed a multi-chamber incubator with controlled temperature and irradiance regimes. The main hypothesis that has been tested is that different environmental stresses lead to specific modifications to the photosynthetic machinery in corals and can be selectively assessed by using FRR fluorometry. In the second year, research was focused on studying the effect of selected anthropogenic stresses, including heavy metal pollution. This component of the project provides background for the development of optical protocols for non-destructive assessment of the health of coral reefs.

Due to the presence of unique pigments in cnidarian hosts, corals are characterized by the amazing variety of colors (Veron, 2000). The diversity of the color palette is an important indicator of the health and viability of coral ecosystems. The major color determinants are proteins homologous to the Green Fluorescent Protein (GFP) from jellyfish *Aequorea Victoria* (Matz et al. 1999; Dove et al., 2001). Thereby, each basic color is essentially determined by a genetic sequence of a single protein (Matz et al., 1999; Labas et al., 2002) that allows for the advanced tools of molecular biology to be directly applied for studying the color diversity of coral reef ecosystems. The molecular biology and genetic part of the project included identification, isolation, as well as spectroscopic, genetic and biochemical characterization of

major color determinants in corals (using the procedures described by Matz et al. 1999). We have used fluorescence spectroscopy and microspectrofluorometry for the preliminary analysis of the color palette in a variety of coral species and for the selection of model species and their color morphs for detailed genetic characterization. We further examined empirical correlation between individual allelomorphs and the photosynthetic and physiological variability retrieved using the FRR variable fluorescence techniques. This part of the research provides scientific background for understanding the optical diversity of coral reef ecosystems and interpreting the results of optical monitoring of benthic communities using spectral fluorescence and reflectance measurements, for example. In addition, the results of this research would potentially accelerate the application of fluorescent proteins from corals for biomedical purposes.

5. PROJECT ACCOMPLISHMENTS

5.1 Instrument development

5.1.1 Fluorescence Induction and Relaxation (FIRe) System for assessment of the physiological status of coral

We designed and constructed a new optical instrument, called Fluorescence Induction and Relaxation (FIRe) System (Gorbunov and Falkowski, 2004). The instrument (Fig. 1) measures photosynthetic characteristics (Table 1) and assess the physiological status of photosynthetic machinery in algae and corals rapidly and in a non-invasive way. The FIRe technique is based on similar biophysical principles as the FRRF and provides the same physiological characteristics. But the optical design has been improved, the electronic circuitries simplified, and the operational protocols extended. This permitted the sensitivity to be enhanced and the production cost to be greatly reduced. To conduct FIRe fluorescence measurements in laboratory aquaria, a watertight fiber-optic attachment has been constructed (Gorbunov and Falkowski, 2004). This system permitted more extensive (compared to previous FRR fluorometers) sampling in the field and also provided the basis for development of underwater submersible fluorosensors for permanent monitoring stations. The bench-top version of the FIRe System has been transferred to a small hi-tech company, Satlantic Inc., for commercial production (see Technology Transfer below).

The FIRe technique relies on active stimulation and highly resolved detection of the induction and subsequent relaxation of chlorophyll fluorescence yields on micro- and millisecond time scales (Fig 2). To accommodate efficient excitation of diverse functional groups within phytoplankton and benthic communities including a variety of cyanobacteria, we have developed a multicolor excitation source. This source uses high luminosity blue (450 nm and 480 nm, each with 30 nm bandwidth) and green (500 nm and 530 nm, each with 30 nm bandwidth) light-emitting diodes (LEDs) to excite chlorophyll and bacteriochlorophyll fluorescence *in vivo*. A computer-controlled LED driver circuitry generates pulses with the duration varied from 0.5 μs to 50 ms. Each LED generate up to 1 W/cm^2 of peak optical power density in the sample chamber or at the leaf surface to ensure fast saturation of PSII within the single photosynthetic turnover (less than 50 μs).

The fluorescence signal is isolated by red (680 nm or 730 nm, each with 20 nm bandwidth, for Chl-a fluorescence) or infra-red (880 nm with 50 nm bandwidth, for BChl-a fluorescence) interference filters and detected by a sensitive avalanche photodiode module. A small portion of the excitation light is recorded by a photodiode as a reference signal. Both the fluorescence and reference signals are amplified and digitized by 12-bit analog-to-digital converters at 1 MHz sampling rate by a custom-designed data acquisition board. To accommodate a wide range of Chl-a concentrations (0.01 to 100 mg/m3) in natural phytoplankton and laboratory cultures, the gain of the detector unit is automatically adjusted over the range of three orders of magnitudes. An embedded low-power Pentium-based board controls the excitation protocols and data acquisition and performs the real-time data analysis using a custom analysis toolbox.

Analysis of fluorescence induction on microsecond time scales (initial part of the FIRe protocol) provides the minimum (F_o) and maximum (F_m) fluorescence yields, the quantum efficiency of photochemistry in PSII (F_v/F_m), the functional absorption cross-section of PSII (σ_{PSII}), and the energy transfer between PSII units ('connectivity factor', p). The recorded relaxation kinetics of fluorescence yields reflects the rates of electron transport on the acceptor side of PSII and between PSII and PSI. The photosynthetic electron transport rates as a function of irradiance, together with coefficients of photochemical and non-photochemical quenching are measured using an incorporated source of background light. The design of the electronic circuitries and operational software are extremely flexible and permit for additional excitation protocols to be implemented, including classical Kautsky induction, the FRR, pump-and-probe, pulse amplitude modulation, and potentially other protocols.

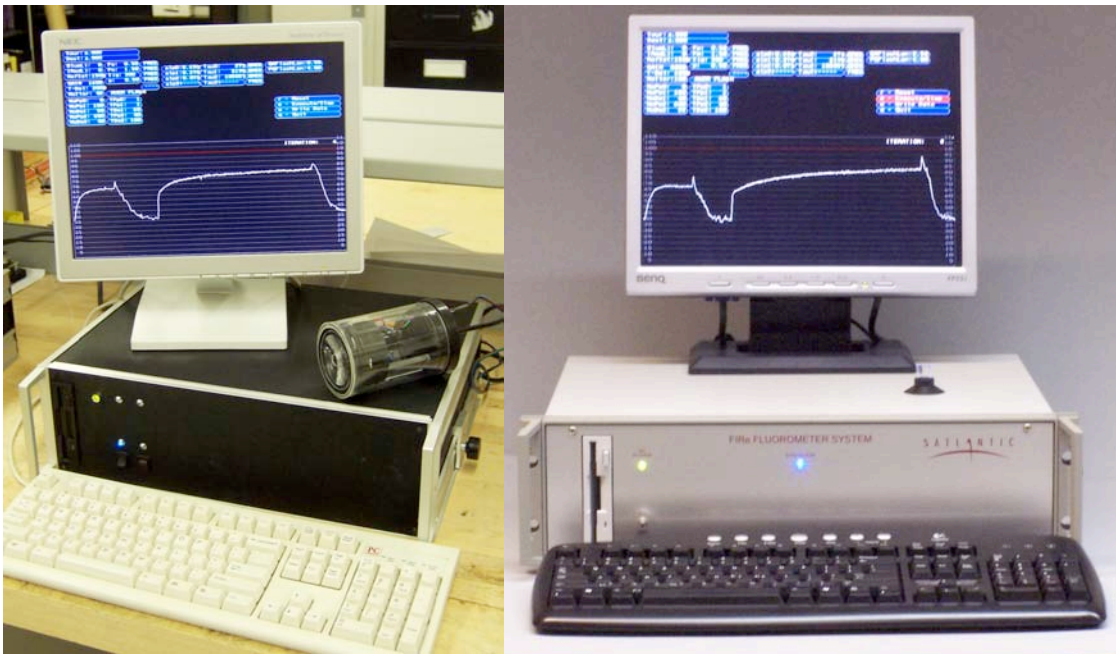


Figure 1. Bench-top versions of the FIRe Fluorometer System. (Left panel) - FIRe System prototype with the watertight optical receiver unit. (Right panel) - Commercial FIRe Fluorometer System manufactured by Satlantic Inc. (see "Technology Transfer" for details).

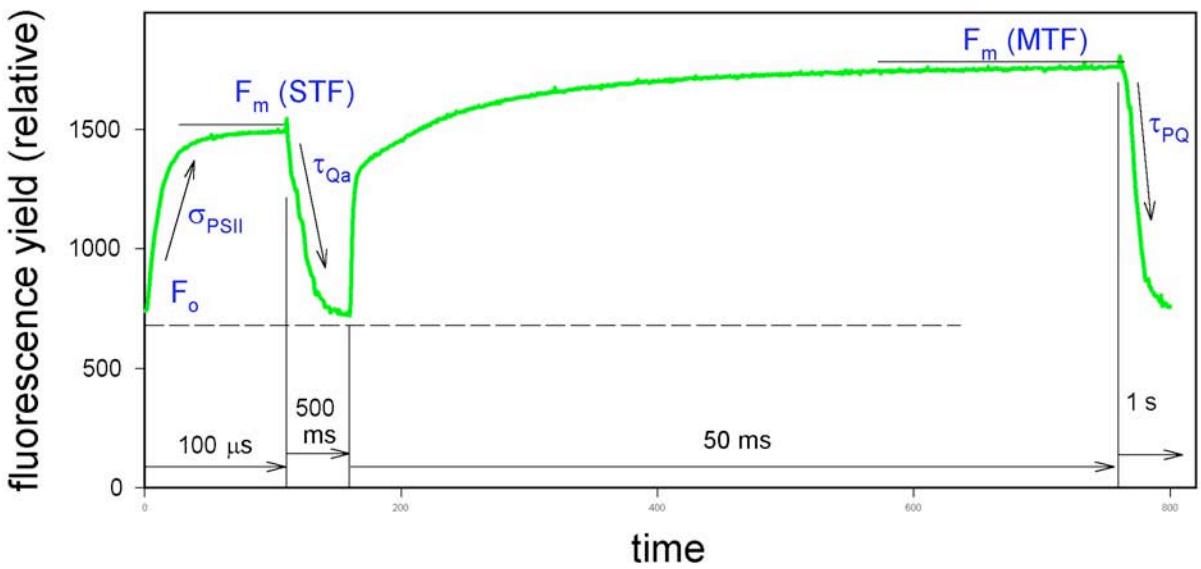


Figure 2. An example of the FIRe measurement protocol consisting of four phases: (1) a strong short pulse of 100 μs duration (called Single Turnover Flash, STF) is applied to cumulatively saturate PSII and measure the fluorescence induction from F_o to F_m(STF); (2) weak modulated light is applied to record the relaxation kinetics of fluorescence yield on the time scale of 500 ms; (3) a strong long pulse of 50 ms duration (called Multiple Turnover Flash, MTF) is applied to saturate PSII and the PQ pool; (4) weak modulated light is applied to record the kinetics of the PQ pool re-oxidation the time scale of 1s. Analysis of the Phase 1 provides the minimum and maximum fluorescence yields (F_o, F_m), the quantum efficiency of photochemical charge separation in PSII, F_v/F_m(STF), the functional cross-section of PSII, σ_{PSII}, and the connectivity factor, p; Phase 2 – time constants for the electron transport on the acceptor side of PSII (i.e., re-oxidation of the Qa acceptor); Phase 3 - F_m(MTF) and F_v/F_m(MTF); Phase 4 – the time constant for the electron transport between PSII and PSI (re-oxidation of the PQ pool).

Table 1. List of fluorescent and photosynthetic parameters measured with FIRe technique

FIRe-retrieved parameter	*Description*
Fo, Fm	Minimum and maximum yields of chlorophyll-a fluorescence measured in a dark-adapted state (relative units)
Fv	Variable fluorescence (= Fm – Fo)
Fv/Fm	Maximum quantum yield of photochemistry in PSII, measured in a dark-adapted state (dimensionless)
Fo', F', Fm'	Minimum, steady-state, and maximum yields of chlorophyll-a fluorescence measured under ambient light, relative units (the prime character indicates the measurements are made under ambient light)
σ_{PSII}	Functional absorption cross section of PSII (A2) in a dark-adapted state
σ_{PSII}'	Functional absorption cross section of PSII in a light-adapted state
ΔF'/Fm'	Quantum yield of photochemistry in PSII, measured under ambient light (=(Fm'-F')/Fm'), dimensionless
Fv'/Fm'	Quantum efficiency of photochemistry in open reaction centers of PSII, measured in a light-adapted state (=(Fm'-Fo')/Fm')
p	"Connectivity factor", defining the exciton energy transfer between individual photosynthetic units (dimensionless)
(Fm- Fm') /Fm	Quantum efficiency of non-photochemical quenching (i.e., light-induced thermal dissipation of excess energy under supra-optimal light
τ_{Qa}	Time constant of photosynthetic electron transport on the acceptor side of PSII (Qa re-oxidation)
τ_{PQ}	Time constant of photosynthetic electron transport between PSII and PSI (re-oxidation of plastoquinone pool, PQ)

5.1.2 Development of underwater multi-receiver FIRe fluorosensor for permanent underwater monitoring stations

Because of the extraordinary diversity of coral reef ecosystems, permanent underwater monitoring stations must be capable of collecting physiological data from multiple targets (e.g., a variety of coral species dominant in the area). This is achieved by the development of a multi-receiver fluorometer with parallel signal acquisition from multiple targets. Therefore, the second stage of the instrument development has been devoted to the design of an underwater multi-receiver FIRe (Fluorescence Induction and Relaxation) fluorosensor for long-term underwater monitoring stations. This system permits simultaneous monitoring of multiple (up to 8) targets.

The first step in this development was design and evaluation of an individual optical detector for the underwater system. We examined two alternative strategies for the fluorosensor design and developed two prototype optical receivers. Both units have been thoroughly tested in the coral

aquariums at Rutgers and iteratively improved. The first model receiver incorporates an individual excitation source and a detector, both being connected to the control unit by a waterproof electrical cable. The cable supplies power, transmits the controlling signals to the excitation source, and delivers the return signal back to the processing unit. The second model receiver relies on the use of watertight optical fibers, with both excitation source and detector being installed in the control unit. The analysis and tests revealed that both approaches work, with the first one being more advantageous and reliable for long-term operation. A watertight optical receiver unit coupled with the FIRe system is shown in Figure 1, left panel.

Another technical task was to evaluate and compare two types of excitation courses for the multi-receiver FIRe fluorosensor for long-term underwater monitoring stations – ultra-bright light-emitting diodes and laser diodes. While the low beam divergence of the laser diodes permitted for the operational distance between the instrument and the target to be increased, the modern diodes (especially blue ones) remain expensive and have relatively short operational lifetimes. During this project, we used red laser diodes (650 nm wavelength, 100 mW optical power) in one of the developed optical heads. Ultimately, we selected the light-emitting diodes (Star V from Lumileds Inc.) as a proven and reliable solution for measurements at short distances. The laser diodes are, however, required for the optical design of the ROV-type of FIRe sensor (sensing at a longer distance, see below). We further developed and incorporated into the system the electronic interface board to support multi-receiver operation and data acquisition. The software package has been developed to support FIRe multi-receiver operation. The developed prototype has been tested in the coral aquarium at Rutgers (Fig. 3).

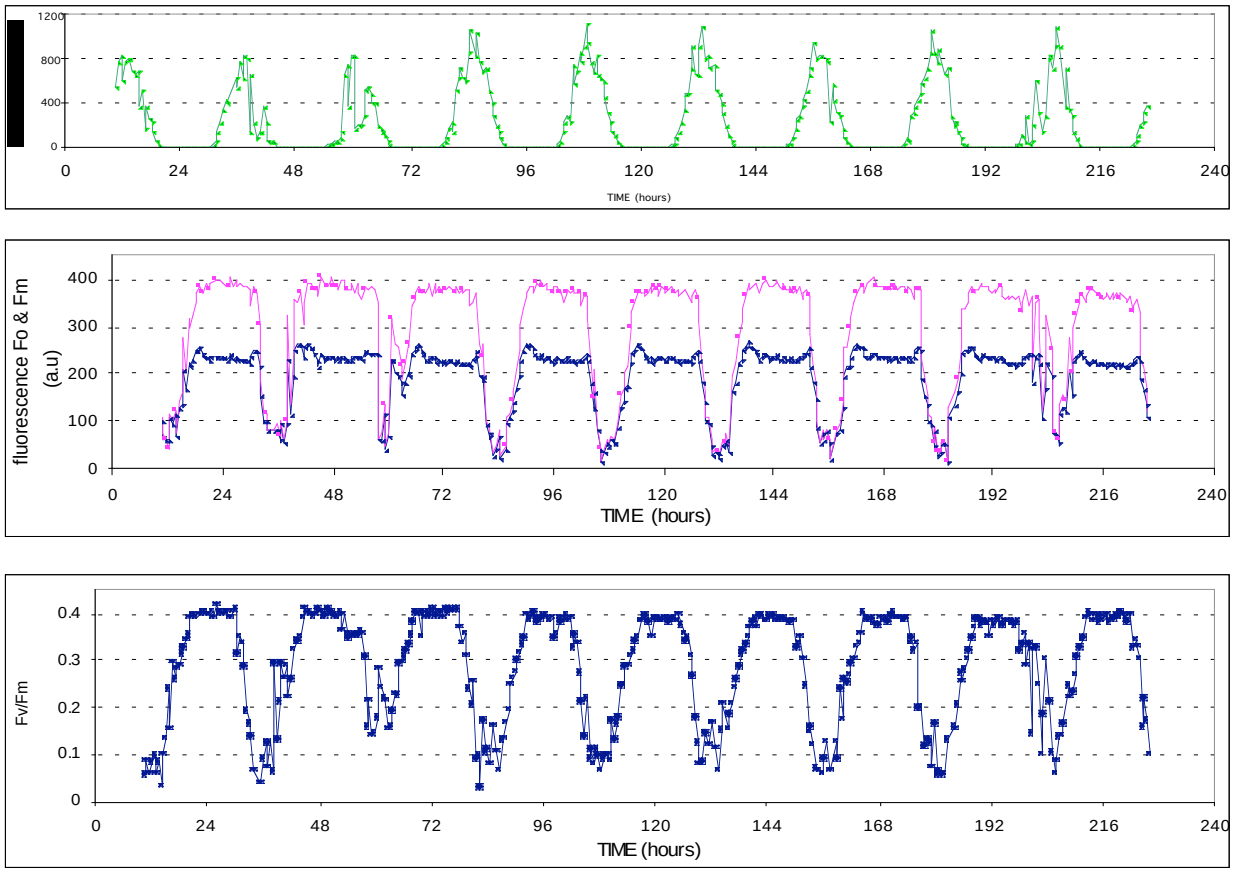


Figure 3. Dynamics of Fluorescence Yields (Fo and Fm, middle panel) and photosynthetic efficiency (ΔF'/Fm', lower panel) recorded over the period of 9 days in the coral *Montipora* sp. by using the developed prototype FIRe system for permanent underwater monitoring stations. The upper panel shows diel variations in ambient irradiance, PAR. Because the physiological state of the coral remained unaltered (non-stress conditions), the diel patterns driven by variations in solar irradiance are precisely reproducible over the 9 day period.

5.1.3 Development and evaluation of laboratory prototype of a FIRe fluorosensor for Remotely Operated Vehicles or Diver Propulsion Vehicles (ROV/DPV).

We have evaluated the potential of FIRe fluorescence measurements using Remotely Operated Vehicles (ROVs) and designed a laboratory prototype of this sensor. The major challenge in developing the ROV/AOV type of instrument was the need to increase the operational distance up to 2 to 3 meters, a typical range of optical sounding in underwater AOV systems. Because the ROV-type of FIRe system utilizes virtually the same electronic package and software routines as the above bench-top FIRe system, the main part of the work was to develop, evaluate and iteratively improve the laser-based excitation source and optical scheme. We approached this task by incorporating high power diode lasers as the source of excitation light. To define the requirements for the laser source, we conducted simulations of the laser beam propagation through the water column, using the known optical properties (spectral bi-directional scatter and

absorption) of natural waters. These simulations are further supported by our measurements in a coral tank (Figure 4).

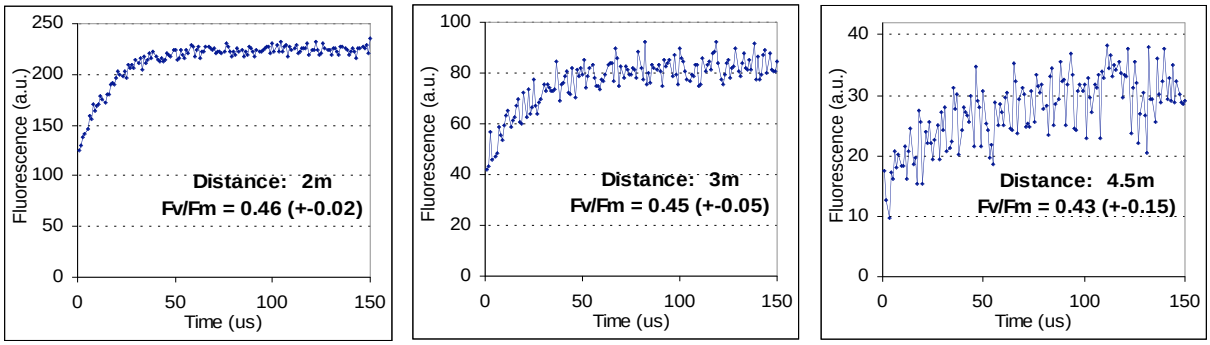


Figure 4. Fluorescence Induction profiles from the coral *Montipora* sp. recorded in a single shot regime by using the laser-based FIRe module designed for ROV/DPV type of carrier. The measurements were made underwater on the same coral at various distances from the sensor to the coral. The calculated photosynthetic efficiencies (Fv/Fm) with standard deviations are shown on each plot. The uncertainty of each measurement is determined by the noise level in the FIRe profiles. Note that at the distance greater than ~ 3m (e.g., right plot) the single shot measurements are too noisy. Accumulation of the signal over multiple shots is therefore required at longer distances.

Based on the measurements in a coral tank, we defined the basic **specifications for a future operational ROV/AOV FIRe sensor**. The data analysis revealed that the peak optical power of the laser pulses must approach ca. 1W to ensure the required optical density at the distance of 3 m in tropical waters (Figure 3). This value, however, must be significantly higher (up to 5 to 8 W) in turbid waters rich of suspended matter. The main factor that limits the operational distance of underwater remote measurements is rapid attenuation of induced red fluorescence by water. Although the laser beam can be easily collimated, the low-angle scatter in water inevitably leads to beam divergence at the scale of a few meters. The most suitable wavelength of the laser radiation was found to be in the blue region (440 to 500 nm). However, modern commercially available blue laser diodes remain very expensive and do not provide sufficient power. At the moment, we selected red laser diodes, emitted at ca. 650 nm, as the best compromise and proven solution for the excitation source. Based on the evaluation and selection of the excitation source, we designed and tested a prototype laser module with high-power quasi-CW laser diode (wavelength 650 nm, peak optical power of 0.5 W). To ensure efficient collection of induced fluorescence, the optical aperture of the lens system was increased to 15 cm (compared with 2 cm in the benchtop FIRe system). Further increase in the aperture would improve the signal-to-noise ratio of the whole system, but it seems to be unfeasible for the size limits of ROV/DPVs. Therefore, the research and development revealed that the above stated characteristics ensure reliable recording (in real-time) of induced fluorescence from coral targets at the distance up to ca 2 to 3 m (Figure 3).

5.1.4 Underwater spectrometer for quantitative characterization of coral fluorescence and reflectance

By augmenting the underwater spectrometry system previously developed in the laboratory of Sensory Ecology, University of Queensland, Australia, we have produced a prototype instrument that is able to take fluorescence and reflectance measurements from corals *in situ* in a highly reproducible manner. Such measurements are essential for quantitative analysis of fluorescence distribution and changes over time. The key parts of the instrument are LED-based source producing blue excitation light for fluorescent measurements and an optical attachment enabling fluorescence measurements at a controlled distance and angle. The same attachment also enables accurate and fast reflectance measurements using ambient light with necessary black and white controls. The small footprint of the attachment (2 cm in diameter) enables measuring individual coral branches or different areas of non-branching species. The new instrument has been successfully field-tested and employed in 2006 at the Looe Key reef, Florida Keys.

5.1.5 Coral Cultivation Facilities

To conduct controlled manipulation experiments in the lab, we constructed Coral Cultivation Facilities at Rutgers University (Figure 5).

Figure 5. Coral cultivation facilities, comprising three 90 gal. tanks with over 20 species of coral. Five smaller (10 gal) tanks with individually controlled temperature (not shown) are used for laboratory manipulation experiments.

5.2 Development of bio-optical algorithms for detection and assessment of the impact of common natural stresses to coral

5.2.1 Effect of natural stresses on symbiotic zooxantellae

A series of experiments have been completed with the aim to elucidate the impact of two common natural stresses (elevated temperature and excess light) on the photosynthetic activity and fluorescence in symbiotic zooxanthellae. The main results of this research are summarized in Tchernov et al. (2004). Thermal stress is the most common stress related to the Global Warming that may be superimposed to specific stresses related to military activity on DoD installations. It is therefore important to understand the mechanisms and signatures of this stress to develop operational protocols for its diagnostics.

The symbiotic algae of the genus *Symbiodinium* spp. (called zooxanthellae) are the key targets in our developed stress detection protocols. Overall, 14 different species of *Symbiodinium* spp. have been tested under both normal and stress conditions. All species have been genetically mapped using direct PCR and sequencing of both the 28 ribosomal subunit and the internal transcribed spacer region (ITS I). These are two major genetic techniques accepted and widely used in plant physiology and phycology. A library of baseline FRR fluorescence and photosynthetic characteristics has been collected for all species. FRR fluorescence measurements were also used to document, with high resolution, the temporal evolution of stress development.

Two groups of zooxanthellae (thermally resilient and sensitive, respectively) have been used for detailed examination of fluorescent and photosynthetic signatures. All examined species exhibit similar responses to thermal stress, with resilient species responding slower and only at higher temperature. Chlorophyll variable fluorescence measurements revealed that the thermal stress leads to a characteristic decrease in Fv/Fm, the maximum quantum yield of photochemistry in Photosystem II (PSII) and a decrease in the number of photosynthetically active reaction centers of PSII (see Fig. 6). The decrease in Fv/Fm is accompanied by a sharp increase in the rate of electron transport on the acceptor side of PSII followed by a decrease in this rate at later stages of the stress development. The first two signatures are general for unfavorable environmental factors (including excessive irradiance and nutrient limitation), whereas the last one appears to be specific for thermal stress.

To elucidate the responses to stress and diagnostic signatures on the cellular to sub-membrane levels, the fluorometric measurements are complemented with optical and electron microscopy, as well as biochemical analysis of the lipid composition of photosynthetic membranes. Microscopic examination of individual zooxanthellae cells revealed that photosynthetic thylakoid membranes of thermally sensitive clones were significantly disrupted under elevated temperature and the organized stacking pattern, which is essential for photochemical energy transduction, was compromised (Fig. 7). These two phenomena are diagnostic of an energetically uncoupled system where the trans-membrane proton gradient, established by the photosynthetic reactions, is dissipated without generating ATP. This pattern is uniquely found in thermally sensitive zooxanthellae algae and differs from photoinhibition (high light stress). These results suggest that thylakoid membrane integrity is potentially a critical determinant of

thermal tolerance in zooxanthellate corals. In contrast, in thermally resilient clones the stacking properties and ultrastructural integrity of the membranes remained unaffected, in consistence with unaltered FRR fluorescence signatures.

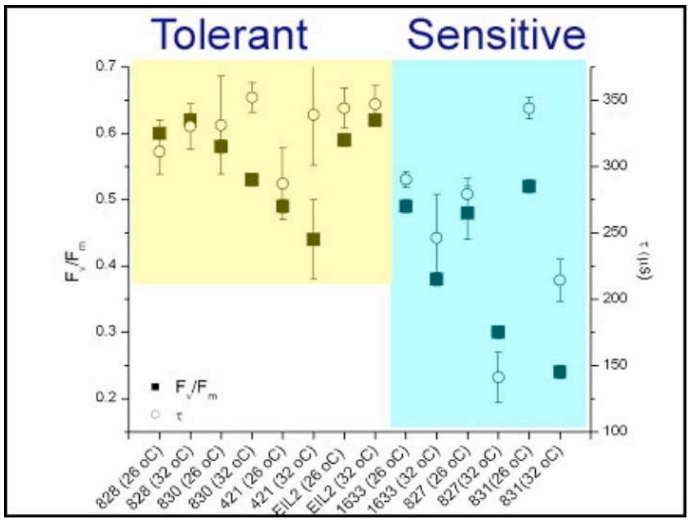


Figure 6. The effect of thermal stress on photosynthetic characteristics of isolated zooxanthellae. Thermally tolerant and sensitive strains were exposed to elevated temperature (32°C). The control samples were kept at 26°C. Note the characteristic decline in the quantum efficiency of photochemistry in Photosystem II (Fv/Fm) and a decrease in the time constant for the electron transport on the acceptor side of PSII in thermally sensitive strains at 32°C.

The results suggest that the stressed algae can be readily diagnosed by using the FIRe fluorescence signatures, even at early stages of stress development. The FRR-derived quantum efficiency of photochemistry in Photosystem II (Fv/Fm) is a primary indicator of stress. Furthermore, the temporal patterns of FIRe signatures under stress development were found to be stress specific that allows for the FIRe fluorescence records to be used for selective diagnostics of the natural stresses. The conducted experiments have also helped in "fine tuning" of our proposed instrumentation and measurement protocols.

5.2.2 Effect of natural stresses on intact coral

Six field trips have been completed in both the Caribbean and Indo-Pacific regions, including two long trips to Hawaii Institute of Marine Biology. The main objective was to elucidate the impact of main natural stresses (episodes of elevated temperature and excess light) on the fluorescent and physiological signatures of coral and collect samples for genetic analysis. The stony corals *Porites compressa*, *P.lobata*, and *Possillopora damicornis* have been selected as model Hawaiian species and represent both thermally sensitive and resilient types. The sensitivity of the corals to thermal stress have been determined from the long-term records of coral bleaching phenomena in the Kaneohe Bay, provided by Dr. P. Jokiel, Hawaii Institute of Marine Biology. Also, a field experiment on the influence of heavy metal ions (Cu and Zn) on

corals was performed in the field, as part of the studies on the impact of anthropogenic stresses and toxins.

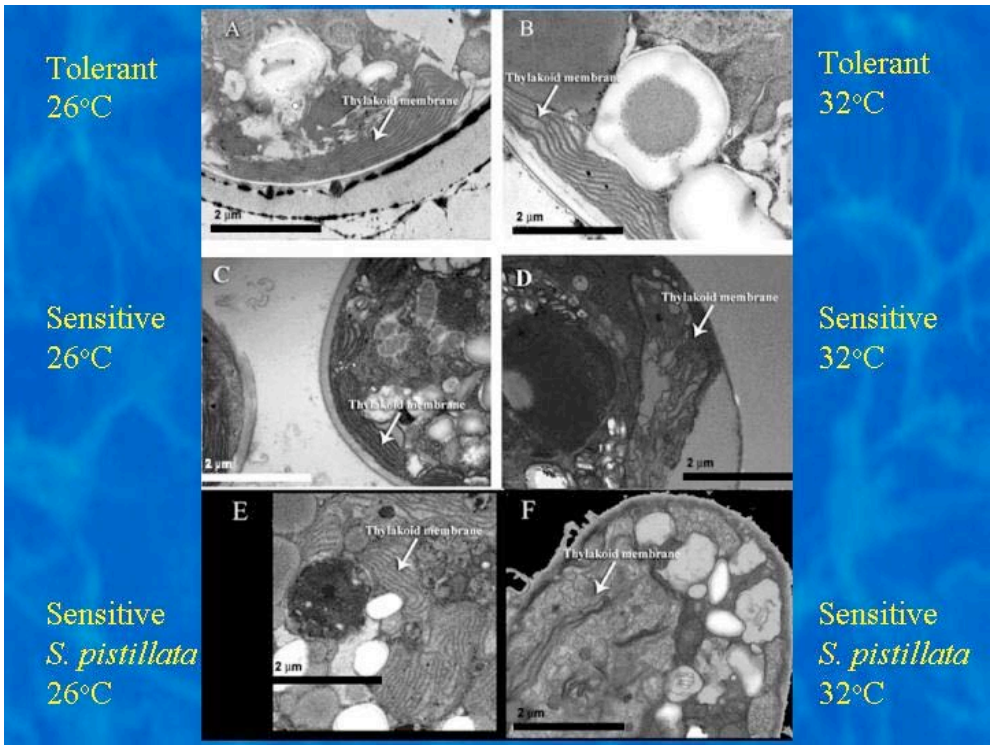


Figure 7. The effect of thermal stress on the structure of individual zooxanthellae cells. Note the ultrastructural integrity and organized stacking pattern of the photosynthetic thylakoid membranes at normal temperature. In contrast, the thylakoid membranes in thermally sensitive clones are dramatically disrupted at high temperature (panels D and F).

The photosynthetic performance of corals *in situ* and in lab aquaria was recorded by SCUBA-based and bench-top FRR fluorometers, respectively. A new watertight fiber-optic attachment for the bench-top FRR fluorometer has been thoroughly tested in coral mesocosms at Hawaii Institute of Marine Biology and employed for the field measurements. Fluorescent microscopy and spectrofluorometry were employed to monitor changes in coral coloration under stress development. Zooxanthellae have been isolated from all studied species for further genetic characterization at Rutgers. Similar to the previous laboratory work on *Symbiodinium* spp., the genetic characterization included direct PCR and sequencing of both the 28 ribosomal sub-unit and the internal transcribed spacer region (ITS I).

The results on intact corals revealed the physiological responses similar to those previously established on isolated algae. The FRR fluorescence showed that the heat stress leads to a decrease in the maximum quantum yield of photochemistry in PSII (Fv/Fm) followed by a long-term reduction in the rate of electron transport on the acceptor side of PSII (Fig. 8). This FIRe-

retrieved pattern is uniquely found in thermally sensitive corals and differs from photoinhibition (high light stress).

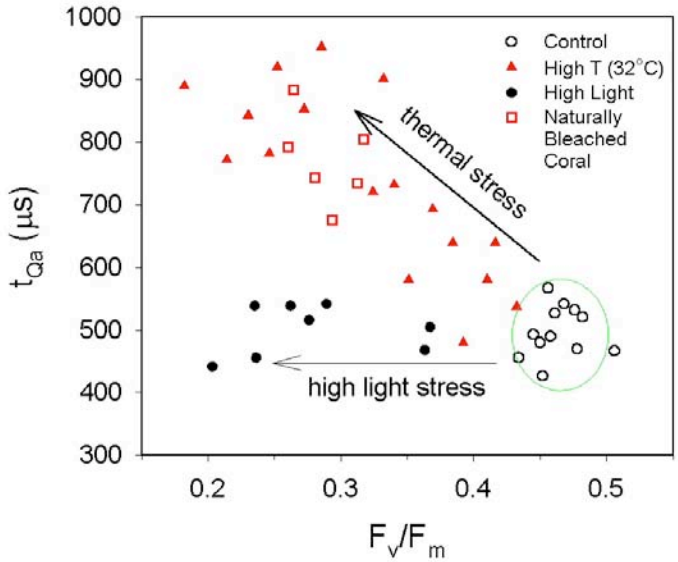


Figure 8. Distinguishing between thermal stress and high light stress (photoinhibition) in coral. The impact of thermal stress and photoinhibition on the photosynthetic characteristics of the coral, *Montastraea annularis*. Both stresses lead to a characteristic decrease in the quantum efficiency of photochemistry in Photosystem II (F_v/F_m), however only the thermal stress results in a striking increase in the time constant, τ_{Qa}, for Q_a reoxidation (Q_a is the first acceptor on the acceptor side of PSII). Also, photoinhibition induced decrease in Fv/Fm is rapidly recovered under low light (within < 24h), while the impact of thermal stress is irreversible. The parameters were recorded by a SCUBA-based FRR fluorometer.

Gas chromatography/mass spectroscopy analysis of five isolates of zooxanthellae, conducted at Rutgers, revealed a striking contrast in the relative composition of lipids associated with thylakoid membranes between thermally sensitive and resilient clones. Specifically, thermally resilient *Symbiodinium* clones have a lower relative content of the major polyunsaturated fatty acid (PUFA), Δ6,9,12,15-*cis*-octadecatetraenoic acid and higher content of Δ9-*cis*-octadecatetraenoic acid. The differences in this lipid profile, which are statistically significant at the 0.001 level, specifically increases in thermal stability in eukaryotic thylakoid membranes, and simultaneously reduces the susceptibility of the membrane lipids to attack by Reactive Oxygen Species. These results strongly suggest that the differential susceptibility to elevated temperature stress in *Symbiodinium* spp. is manifested in lipid biosynthetic pathways not associated with lipid desaturases per se, but rather with regulatory elements of the enzyme(s) that control(s) the relative amount of desaturation in specific pools of fatty acids (Tchernov et al, 2004).

5.3 Role of Programmed Cell Death (PCD) in death of bleached corals

The environmental factors that induce coral bleaching (the loss of the coral's photosynthetic symbionts) include oxidative stress, an outcome of both elevated temperatures and high light. We set out to determine the physiological and biochemical pathways that result in the coral's death after bleaching, and to test the sensitivity of different species to oxidative stress. Specifically, we set out to establish whether the coral death is caused by Necrosis (accidental death) or Programmed Cell Death, PCD, (genetically regulated). These two processes have different morphological, physiological and biochemical characteristics. In PCD, but not in necrosis, a family of cysteine proteases, Caspases, mediates the cellular changes that lead to the cell's death. Therefore, monitoring the caspase activity may serve as a diagnostics of coral stress and potentially be used to predict the fate of stress development. Using a fluorogenic caspase substrate we were able to monitor caspase activity in corals exposed to oxidative stress induced by elevated temperatures, or artificial induction.

5.3.1 Lab Studies

Experiments were set up at the coral lab of the New York Aquarium and then continued at the Rutgers coral facilities. Two coral species, *Stylophora sp*. (thermally sensitive), and *Montipora sp*. (more resistant) were incubated at elevated (32°C), and control (26°C) temperatures. In *Stylophora sp*., increased caspase activity was measured at 32°C, but not 26°C. *Montipora* from both 32°C and 26°C treatments exhibited only background levels of caspase activity. Electron Microscope images showed the characteristic PCD morphological changes in *Stylphora* cells under thermal stress (32°C). Cell lysates were challenged with a caspase antibody, and the cross-reaction indicated that there was more enzyme present in the lysate of sensitive coral from elevated temperature. In the 32°C tank, coral of the sensitive species was bleached within a few days and its tissue disintegrated soon after, while the resilient species was bleached with the tissue remaining intact for at least two weeks.

5.3.2 Field Work

A three-week long field trip to the Hawaii Institute of Marine Biology, on Coconut Island, Kaneohe Bay, Oahu, was organized and conducted in February 2004. Two model species were used for field experiments, *Pocillopora damicornis* (thermally sensitive to bleaching), and *Montipora patula* (more resilient). A series of incubations was set up under different conditions: ambient temperature under high and low irradiance, and two elevated temperatures, 30°C and 32°C, at the same light regime. The results are summarized as follows:

(1) Under ambient temperature, in both light intensities, specific caspase activity remains at background level. In contrast, incubation at 32°C triggered higher caspase activity in high light for both species (although the specific activity of the resilient *Montipora* was lower than *Pocillopora*). *Pocillopora* also showed higher activity at low light intensity.
(2) The induction of increased activity in *Pocilopora* was very rapid (> 5 times in only 6 hours).
(3) Higher concentration of reactive oxygen species (ROS) is observed in corals incubated at 32°C which indicates that the corals are under oxidative stress.

(4) Artificial treatment with ROS (0.1 mM $FeSO_4$/0.6 mM H_2O_2) also resulted in increased caspase activity, in both species.
(5) Artificial treatment with ROS results in higher caspase activity in corals containing zooxanthellae, compared to corals lacking zooxanthellae (i.e., bleached by extended incubation in the dark).
(6) Corals lacking zooxanthellae exhibit only background caspase activity while exposed to elevated temperature (32°C), suggesting that zooxanthellae, but not Cnidarian hosts, are the target of thermal stress. The calculated contribution of the zooxanthellae to the stress inducing PCD is at least 85%.

These results suggest that:
(1) Hermatypic corals experience oxidative stress under elevated temperature and high light, thereby the majority of reactive oxygen species is produced by the symbiotic zooxanthellae.
(2) When exposed to these stress conditions, the cells induce a PCD response, as indicated by increased activity of PCD specific proteases, caspases.
(3) The caspase activity mediates cell death of bleached corals.
(4) The sensitivity to a specific stress is species dependent, which results in different rates of PCD induction. The results obtained here, however, cannot explain the difference in response between different species.

Therefore, the results of this work suggest that the animal host cells are genetically programmed to either activate the caspase cascade (in which case, elevated temperatures can lead both to bleaching and death of the animal host), or not (in which case, elevated temperatures can lead to bleaching, but not death of the animal). The resulting combinatorial genetic matrix provides a basic diagnostic function for coral bleaching (Tchernov et al. (2007).

5.4 Study the impact of selected anthropogenic stresses on coral.

Metal contamination is one of the main anthropogenic stresses on coral reefs worldwide. The contamination sources include anti-fouling paints, sewage, industrial and agricultural run-off. Early detection of metal poisoning is essential for development and implementation of the ecosystem protection and remediation programs.

This part of this SERDP project focused on (1) elucidating the physiological mechanisms of stress, (2) developing a method of recording the physiological response of corals to metal contamination, (3) finding biomarkers to diagnose the response of coral to metal contamination.

Using our coral cultivation facilities, we examined the role of heavy metal contamination (copper and zinc) on the photosynthetic characteristics of zooxanthellae, Reactive Oxygen Species (ROS) generation, and caspase regulation in coral host. Our experiments revealed that the animal host of coral symbioses is much more susceptible to toxic metals than algal symbionts (Fig. 9). Metal exposure greatly enhanced caspase activity (an indicator of Programmed Cell Death, PCD) in the animal host followed by coral tissue degradation. The detrimental change was recorded prior to any decline in the photosynthetic efficiency of zooxanthellae (Fig. 9C). The revealed signatures of the heavy metal stress differ dramatically from the impact of common natural stresses

(elevated temperature stress and photoinhibition). Although the operational protocols to diagnose the heavy metal stress remain to be implemented, the results suggest that this stress is distinguishable from the common natural stresses.

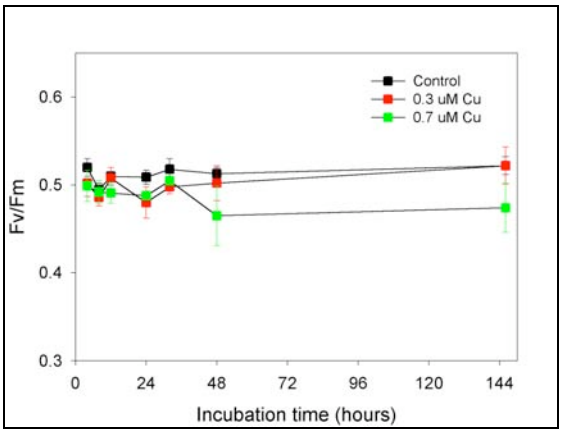
A

control

B

0.75uM Cu, 24 hrs

C



D

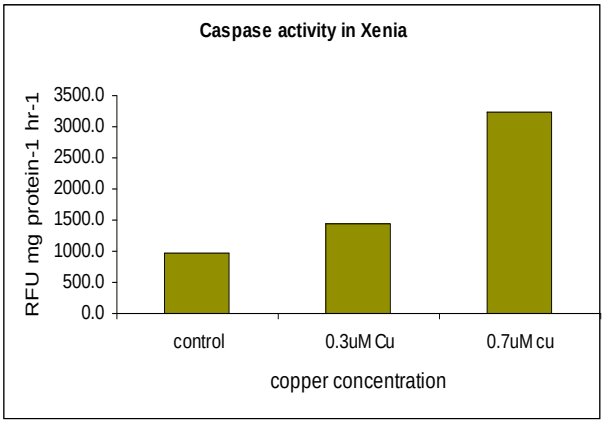


Figure 9. The effect of copper exposure on the symbiotic coral, *Xenia elongata*. The copper poisoning is evident from the detrimental change in polyp behavior (panel B versus control, panel A) and the apoptotic response in the coral host (recorded as elevated Caspases activity) (panel D). The latter was measured as the activity of Caspases (panel D), a family of proteases that are only activated when PCD is induced. Note, that the photosynthetic activity of algal symbionts (Fv/Fm) remain high even at the late stages of the stress development (panel C), suggesting that coral host is more susceptible to the copper stress than the algal symbionts.

To develop a biomarker for heavy metal stress, we focused on early detection of the induction of small metal binding proteins, metallothioneins, in coral tissue. Metallothioneins are found in all metazoans and are important in intracellular regulation and detoxification of metals. These proteins are induced by elevated metal concentration in the environment. As an example, detection of metallothionein is used as a biomarker for metal contamination in mollusks.

We employed several approaches to directly record the level of metallohtionein in coral tissue after exposure to different concentrations of copper. The results show that monobromobimane, a labeling compound that binds to thiols and is used for detection of metallohtionein, is not sensitive enough when samples were run on SDS-PAGE gels. When the labeled samples were run through an HPLC column, a small molecular weight protein was detected. To visualize this protein on a gel, we ran tissue extracts on a 16.5% Tris-tricine gels. These gels are specific for separation of small molecular weight proteins. A low MW (10kD) band was found in the treated samples but not in a healthy coral extract. The ability to separate a protein that may be a metallohtionein on the gel allowed us to further investigate it in two ways. First, we obtained an antibody for Pacific oyster metallohtionein and tested it (on a Western Blot) against the extract of coral tissue that was incubated with copper. The antibody cross-reacted in all the samples that contained high molecular weight protein(s), suggesting that this is a non-specific binding and the mollusk antibody doesn't recognize the coral's metallohtionein. Second, the tissue extracts of copper-treated *Xenia* were HPLC purified, using Sephadex G-50. The low molecular weight fraction was sent for N-terminal sequencing. Of the 18 amino acids at the N-terminal end of the collected protein, three are Cysteines, two of them are one amino acid apart, which is a typical motif in metallothioneins.

Based on the sequence of this protein, we designed new sets of primers that were used in PCR reactions on DNA from two species of corals, *Xenia elongata* and *Pocillopora damicornis*. We got amplification of DNA from several pairs of primers and are now in the process of sequencing these genes. The sequenced *Xenia* protein may be used to make an antibody to detect the protein in coral tissue.

5.5 Optical identification of benthic organisms and assessment of coral coverage on reefs

With the aim to identify optical signatures of different groups of benthic organisms and to evaluate optical protocols for their identification, we collected and analyzed over 3,000 measurements of fluorescent and photosynthetic signatures of different benthic organisms. We tested the hypothesis that different benthic organisms may have specific suites of fluorescent and photosynthetic signatures that may allow us to develop operational protocols to distinguish between different functional groups.

We employed our SCUBA-based Fast Repetition Rate (FRR) fluorometer and Fluorescence Induction and Relaxation (FIRe) system to record chlorophyll fluorescence yields and to retrieve a suite of photosynthetic characteristics of the organism. During data analysis, the benthic targets were clustered into four major functional groups: corals, macroalgae, seagrass, and algal turfs. Our particular focus is given to assessing the percentage of coral coverage on reefs that is an important ecological indicator of the reef viability. The analysis revealed that fluorescence yields are highly variable within each functional group and overlap between the groups, thereby the fluorescence yields alone do not allow one to distinguish between different targets. In contrast, fluorescence-based photosynthetic parameters (the photosynthetic efficiency, Fv/Fm, and the functional absorption cross sections of Photosystem II, Sigma-PSII) are much more conserved and specific for each individual group. Different benthic organisms were found to exhibit unique sets of FIRe-derived fluorescence and photosynthetic signatures that can be

employed for identification of benthic targets. Corals exhibit highly conserved photosynthetic efficiency (Fv/Fm in the range from 0.42 to 0.50) and functional absorption cross sections. Algal turfs have the largest cross sections and high photosynthetic efficiency (Fv/Fm ~ 0.5). Seagrasses exhibit the smallest cross sections and very high photosynthetic efficiency (Fv/Fm = 0.65 to 0.70), with the exception of senescent and dead leaves. Macroalgae are characterized by the highest level of variability in both fluorescence yields and photosynthetic efficiency, attributed to environmental variations in the nutrient status of these benthic organisms. The cluster analysis revealed that the four major groups of benthic communities are readily identified based on FIRe-based fluorescent signatures.

5.6 Ecology of coral colors.

Rationale: Proteins homologous to the Green Fluorescent Protein (GFP) are the major color determinants in reef-building corals and thus represent an easy target for optical non-invasive coral monitoring. Provided that we know the particular functions of the diverse colors together with molecular and/or ecological mechanisms of their expression regulation, it would be possible to obtain valuable information concerning present condition, genetic affiliation and/or individual life history of a coral by spectroscopically analyzing the relative expression of the fluorescence determinants in the field.

5.6.1 Cloning and characterization of color proteins from corals

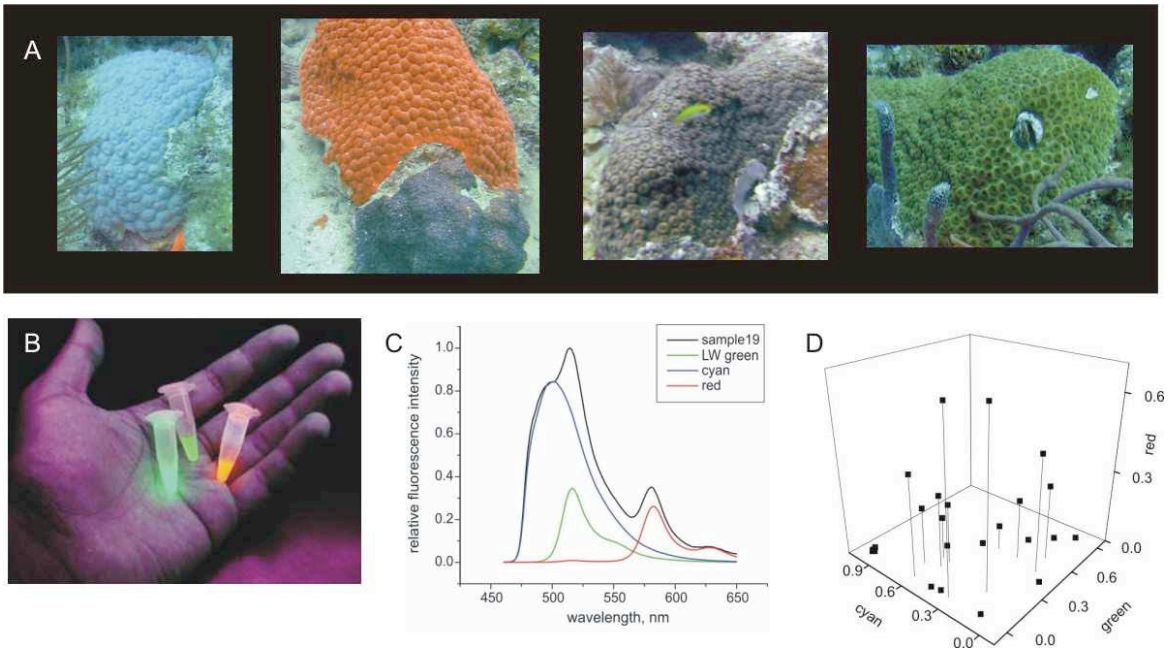


Figure 10. Coral color quantification based on the knowledge of individual color determinants. A: appearance of some of the sampled great star coral colonies *in situ.* B: Tubes containing pure cyan, green and red pigments of the great star coral, cloned and expressed in bacteria, under UV-A light. C: Interpreting the in vivo fluorescence spectra (black curve) as a sum of varying contributions of individual pigments. D: "Color space" describing 28 sampled colonies, showing relative contributions of individual pigments. The color difference between the colonies can be calculated as the distance between the dots representing their color.

The knowledge of spectroscopic characteristics of individual color determinants in corals is essential for the development and "fine-tuning" of optical monitoring instrumentation and protocols. We have completed cloning, expression and characterization of 30 individual fluorescent and colored proteins in corals belonging to 14 out of 21 existing families of reef-building corals. For the key reef-building families (Acroporidae, Faviidae, Poritidae) multiple pigments per organism have been identified. On the basis of this knowledge, a methodology for rigorous quantification of color change and polymorphism has been developed (Fig. 10). The results revealed that similar to the previously characterized Caribbean coral, *Montastraea cavernosa*, the Indo-Pacific corals contain multiple genes of fluorescent proteins coding for three basic colors: cyan, green and red. The fourth identified gene type, present in the *Acropora* but not found in the Caribbean corals, codes for non-fluorescent purple-blue coloration. The results of this work are summarized in Ugalde et al. (2004), Field et al. (2006), and Lukyanov et al. (2006).

Based on our genetic and spectroscopic characterization of coral color proteins, we assessed feasibility of discriminating alleles of coral fluorescent proteins spectroscopically. As it was

found recently (Kelmanson and Matz, 2003), color polymorphism in corals is due primarily to differences in gene expression levels rather than mutations in coding regions of different alleles. Still, the altered gene expression level can be a signature of an allele with mutated regulatory (rather than coding) gene region. To sort this out it is necessary to determine how heritable the color is, by crossing the color morphs. We tried to do this experiment but failed due to coral spawning schedule breakdown in 2005. In most corals, the spawning occurs only during a few days a year, with the exact timing being impossible to be predicted. During the SERDP project we also pursued an indirect way to assess color heritability - by developing genetic markers based on the fluorescent protein genes and trace their distribution in the color morphs. For the Caribbean model coral, *Montastrea cavernosa*, we looked for polymorphisms in introns of the color-coding genes and (in the case of red protein) also at the promoter region. However, the research revealed multiple (3 or more) very similar genes per organism coding for each of the basic colors (Field et al, 2006; Lukyanov et al, 2006). In this situation, the spectroscopic discrimination of alleles would be very problematic if possible at all.

5.6.2 The evolution and function of coral color diversity

We have demonstrated that the fluorescent color diversity in reef corals evolved via natural selection which may indicate that the color serves an essential function in corals' survival.

The **history of color evolution** in one of the major coral groups – suborder Faviina – has been reconstructed to the level of inferring and actually recreating the extinct ancestral pigment variants (Fig. 11), which established that all coral colors descended from a "shortwave-green" ancestor (Ugalde et al, 2004). Statistical phylogenetic analysis then revealed that both instances of new color appearance were accompanied by episodes of natural ("Darwinian") selection, indicating evolution of new essential functions. Finally, site-directed mutagenesis experiments proved that the parts of the pigment that were evolving under selection were indeed the ones responsible for generating the new colors (Field et al, 2006).

Figure 11. Phylogenetic tree of the coral fluorescent proteins drawn on a Petri dish with bacterial cultures expressing resurrected ancestral proteins and present-day proteins.

Natural color polymorphism was found to be unrelated to the genetic background of the algal symbionts residing in the corals' tissues.

We tested this hypothesis on two coral species - great star coral *Montastrea cavernosa* in the Caribbean and staghorn coral *Acropora millepora* in the Indo-Pacific. For both of these species a full complement of pigments has been cloned, which made possible the quantitative color comparison between morphs as described above. We measured and genotyped 28 colonies of the great star coral and 14 colonies of staghorn coral. Neither of these cases supported our hypothesis. Although a notable genotypic diversity of symbionts was detected in both cases, there was no correlation with the color of the host.

Different coral pigments may function as either a warning or a camouflage when perceived by the reef fishes in the natural environment.

We collected the spectral data on the coral color appearance using the underwater spectrometer. We modeled the fish vision response to the coral colors in the natural lighting conditions. The results suggested the possibility of two novel functions of the coral colors: (i) strong visual signaling as a warning (aposemantic) coloration; and (ii) masking the color of symbiotic algae within the coral tissue as a protection from herbivorous fishes (Matz et al, 2006).

5.6.3 Impact of environmental stresses on spectral fluorescence and absorbance

We have examined the effect of long-term thermal stress and acute heavy metal poisoning on a model coral, the ubiquitous Indo-Pacific coral, *Porites lobata*. In both cases the spectroscopic characteristics of the coral changed perceptibly (Fig. 12). Nevertheless, the analysis revealed that the spectral fluorescence and absorbance alone do not carry enough information to enable classification of the stress. Also, the change in spectral reflectance (due to paling) was recordable at later (compared to the detrimental change in FIRe-deduced photosynthetic efficiency, see section **5.2**) stages of stress development. This is primarily because the effects of the same stressors were not readily reproducible from colony to colony. The greatest difference in response was observed between different color morphs. The data suggest that spectral fluorescence and absorbance of the live coral alter under different types of stress, but these changes alone are unlikely to become a good signature to quantify and classify stress causes.

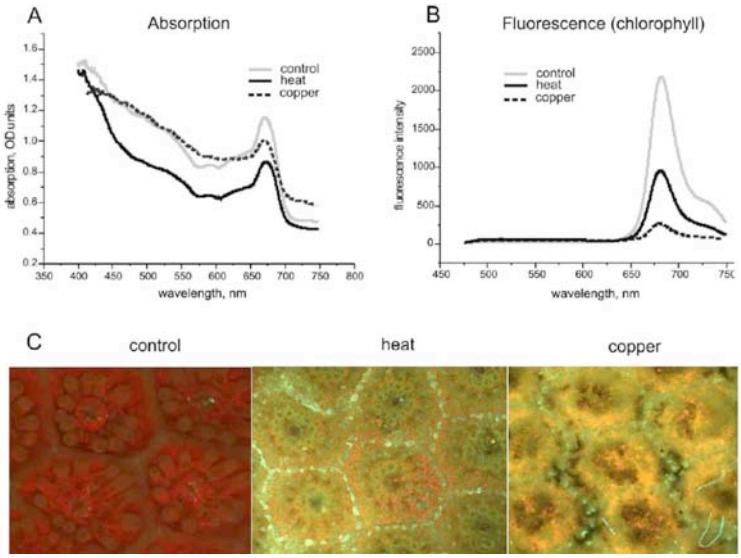


Figure 12. Effects of elevated temperature and presence of CuSO4 on the coral, *Porites lobata*. (A) Absorbance of the coral surface (deduced from reciprocal reflectance). (B) Spectral fluorescence under excitation at 450 nm (chlorophyll band). (C) Appearance of the polyps under fluorescent stereomicroscope.

We therefore focused on two complementary ways to further approach the goals of the project:

(1) To develop a molecular technology for coral stress assessment based on functional genomics. This technology, although not readily applicable for field surveys, would serve as an essential benchmark for any other forthcoming technique.

(2) To study functions and factors affecting the distribution of coral colors, to enable the use of optical techniques to get insight into the ecological processes at particular reefs. Such knowledge would enable predictions concerning long-term resistance and resilience of the reefs.

5.7 <u>Functional genomics of coral stress and fluorescence</u>

In order to establish a molecular method for detection and quantification of stress in corals, we aim to identify genes that change their expression level under stress conditions (see Fig. 13). Such genes are used to prepare an array that can be hybridized to cDNA from the corals under investigation to quantitatively assess the level and nature of stress. Although the technique would require invasive sampling, it is essential as a benchmark while developing new non-invasive optical methods. Also, we are comparing gene expression levels between areas of the same coral colony that exhibit different fluorescence, to understand the nature of cellular processes underlying the change in fluorescence.

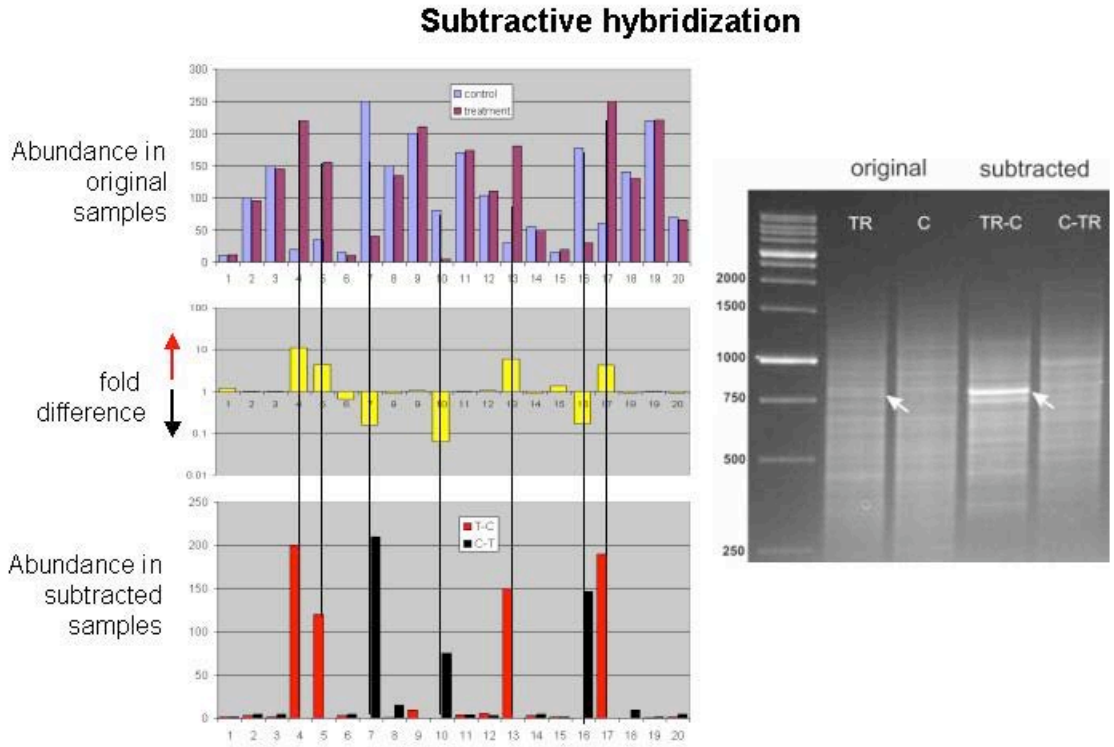


Figure 13. Hunting for stress-specific genes in coral. *Left panels*: cDNA subtractive hybridization transforms cDNA samples so that only cDNAs of the genes that have changed their expression levels during experiment (up-regulated, red arrow on the middle left panel; or down-regulated, black arrow) remain abundant. The procedure shows how the abundances of 20 cDNA species changes as a result of subtractive hybridization, depending on the difference in their abundance in the original samples. The resulting abundance depends primarily on the degree of difference and, to a lesser extent, on the original absolute abundance of the transcripts. The subtracted samples are cloned in bacteria and are being screened for clones that contain differentially expressed genes (a procedure called "differential screening"). *Right panel*: agarose gel electrophoresis of original and subtracted cDNA samples from heat / light stressed coral *Porites lobata* (treatment, "TR") and unstressed coral (control, "C"). Arrows mark one particular fragment that was detected as TR-specific. First lane is molecular weight standards, the corresponding fragment lengths are given near the bands.

A cDNA array based on three two-way subtractive hybridizations has been manufactured containing genes that are up- or down-regulated during stresses (heat, copper poisoning or mechanical injury). By examining the expression of these genes we would derive a statistical method for classification and quantification of natural stress factors, as well as gain insight into evolution of stress-related gene networks in corals. Thus far, we have studied heat/light stress, copper toxicity and mechanical injury using Suppression Subtractive Hybridization (SSH) and identified 125 differential cDNAs. Our results prove that gene expression can serve as a sensitive

quantifier and classifier of stress factors (Fig. 14). However, the identification of the stress-specific genes requires further work.

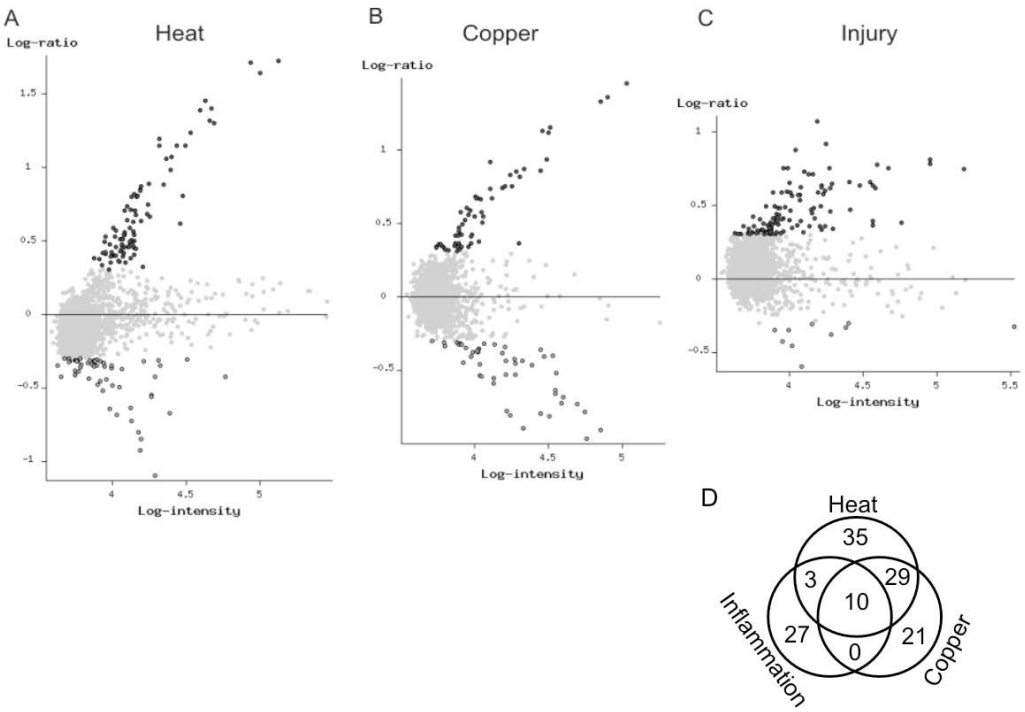


Figure 14. Results of the first round of the array hybridizations. *Panels A-C*: Scatter plots of the intensity ratio between two compared samples at each spot versus mean spot intensity, in logarithmic coordinates. Spots significantly deviating from the mean are highlighted: the ones above zero are up-regulated, the ones below - down-regulated. The plots were generated using the NIA Array Analysis tool [http://lgsun.grc.nia.nih.gov/ANOVA/]. *Panels D:* breakdown of the pool of the 125 significantly differentially expressed sequences.

6. TRANSITION PLAN

We completed technology transfer of the bench-top FIRe (Fluorescence Induction and Relaxation) System to a small hi-tech company, Satlantic Inc. for commercial production (see www.satlantic.com/fire). This system can be used in a wide range of scientific and environmental applications, including water quality monitoring, assessment of primary production and health of aquatic ecosystems, assessment of environmental stresses in marine and terrestrial plants, ecophysiology, photosynthesis research, forestry and agriculture. In addition to this SERDP project, our custom FIRe systems have been employed during 8 oceanographic expeditions in the Atlantic and Pacific Oceans to study photosynthesis and primary production.

Together with Dr. Oscar Schofield, Rutgers University, we are now working on the NOPP (National Oceanographic Partnership Program) funded project to develop a small, low power-consuming FIRe sensor for Gliders (autonomous underwater vehicles). This system is designed for environmental monitoring of primary production in coastal and open ocean waters. We also envision that this new sensor will be also modified for inclusion into a compact underwater sensor for vertical profiling of the water column. Satlantic Inc. already expressed its interest in commercializing this type of sensor.

We envision that our technology will be integrated in a long-term multi-platform monitoring program. While remote sensing and hyperspectral imaging may provide coverage at meso- and potentially global scales, FIRe sensors provide detailed characterization of the physiological status of aquatic ecosystems at micro- and mesoscales.

7. CONCLUSIONS AND RECOMMENDATIONS

The conducted research and development provide a comprehensive set of quantitative baseline data, as well as advanced methods and ready-for-transition technology needed for the assessment of benthic ecosystems near DoD installations. We designed and developed a set of optical instruments, called Fluorescence Induction and Relaxation (FIRe) systems to measure the photosynthetic characteristics and to assess the physiological status of benthic organisms including coral. Together with spectral fluorescence and novel molecular biology techniques, this provides comprehensive and detailed assessment of DoD coral reef ecosystems. The research revealed that the developed method is very sensitive to changes in the coral physiology and records detrimental changes at early stages of the stress development - before any visible changes in coral coloration appear. Because the technique records an extensive suite of physiological parameters, there is also a possibility to diagnose what kind of stress is involved. For instance, the developed protocols were successfully employed to distinguish between common natural stresses (thermal stress and photoinhibition) and selected anthropogenic stresses, such as metal toxicities.

The FIRe fluorometry is, in essence, based on the same biophysical principles, as several other active fluorescence techniques (e.g., pump-and-probe or PAM fluorometry), but it provides significantly greater quantity of parameters (Kolber et al. 1998; Gorbunov and Falkowski 2005). This information is extremely valuable in the identification and quantitative assessment of specific environmental stresses (e.g., elevated temperature, excess irradiance, nutrient limitation, etc.). By analogy with active fluorescence techniques applied to phytoplankton and terrestrial vegetation, we anticipate that the application of FIRe technology will help identify and distinguish between natural and anthropogenic stresses to benthic organisms.

The developed bench-top FIRe instruments are used for analytical measurements on samples or, using fiber attachments, in coral tanks. SCUBA-based instruments are designed for diving operation and survey. Moorable versions of this instrument would be designed for long-term measurements at permanent underwater stations. Our SERDP research suggests that the best strategy for operational use of this technology would be production and installation of underwater systems for permanent underwater stations at several locations with continuous

recording and analysis. Such measurements are conducted in a fully automatic mode and would alert marine biologists and environmental policy makers as to potential detrimental changes in the coral.

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9. PUBLICATIONS

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