ADDENDUM TO THE FINAL REPORT

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

SERDP Project RC-1334

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Maxim Y. Gorbunov
Paul G. Falkowski
Institute of Marine and Coastal Sciences

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List of Acronyms

AOV	Autonomous Operated Vehicle
DSV	Diver-Swam Vehicle
HL	High Light
FIRe	Fluorescence Induction and Relaxation technique
FRR	Fast Repetition Rate fluorometry
LED	Light Emitting Diode
LL	Low Light
MTF	Multiple Turnover Flash
PAM	Pulse Amplitude Modulation
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
ROS	Reactive Oxygen Species
ROV	Remotely Operated Vehicle
STF	Single Turnover Flash
YBD	Yellow Band Disease

Keywords

Environmental monitoring,
benthic ecosystems,
coral reefs,
metal toxicity,
petroleum toxicity,
natural and anthropogenic stresses

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1. Abstract

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 of particular stressors.

Objective: This project is aimed at developing advanced bio-optical techniques and algorithms for rapid and non-destructive assessment and monitoring of coral reef communities, and having capabilities to identify and quantify natural and anthropogenic stressors.

Technical approach: The novel bio-optical technology, called Fluorescence Induction and Relaxation (FIRe) technique, has been developed to measure a comprehensive set of photosynthetic and physiological characteristics of the organism. The measured parameters characterize the excitonic energy transfer in the photosynthetic light-harvesting antennae, the photochemistry in Photosystem II (PSII), and the photosynthetic electron transport to carbon fixation. Because these processes are particularly sensitive to environmental factors, the FIRe technology provides the basis for diagnostics and identification of natural and anthropogenic stressors.

Results: This technology has been employed in laboratory and field studies to establish the physiological mechanisms and fluorescence signatures of common natural factors (such as thermal stress and bacterial diseases) and anthropogenic stressors (metal and petroleum toxicity) on coral. The research revealed that different environmental stressors lead to specific modifications to the coral physiology and are characterized by unique combinations of FIRe fluorescence signatures. The common natural stressors were found to affect the efficiency of primary photosynthetic reactions in PSII. Thereby, the bacterial attack starts with a detrimental change in the photosynthetic electron transport in PSII, whereas the thermal stress impairs both the electron transport and the quantum efficiency of PSII. In contrast, poisoning by heavy metals (such as Cu, Zn, Pb, Sn, and Cd) starts with impairment of the secondary photosynthetic reactions and cell growth. Poisoning with petroleum products is characterized by a specific short-term (minutes to hour) response followed by long-term (days) impairment of photosynthesis. The short-term response exhibits a unique set of FIRe fluorescence signatures, including an immediate decline in the efficiency of the primary photosynthetic reactions and the optical cross-sections of PSII, as well as a dramatic reduction in the rates of electron transport. This response is indicative of rapid absorption of petrochemicals by the cellular lipid membranes, followed by slower penetration of petroleum into the protein complexes. The discovered characteristics and diagnostic signatures of petroleum toxicity differ strikingly from those of other stressors (such as nutrient load, thermal stress, or heavy metal toxins). On this background, rapid bioassay protocols for diagnostics of petroleum toxicity have been developed that are 10 fold more sensitive, compared to previous fluorescent methods.

Benefits: This research has provided the background for the development of bio-optical algorithms for non-invasive detection and assessment of natural and anthropogenic stressors. It is envisioned that the developed technology will be incorporated into long-term environmental monitoring programs, both military and civil, for the assessment and monitoring of aquatic and terrestrial ecosystems.

2. Project Objectives

This SERDP project has been designed to serve the basic objectives of SON CSSON-03-02 "Assessment of Benthic Communities for the Department of Defense". Development of advanced technologies for monitoring and assessing the coral reef communities is a critical component of establishing baseline environmental data at DoD installations and a primary goal of the SON CSSON-03-02. Executive Order 13089 "Protection of Coral Reefs" directs Federal agencies including DoD to study, restore, and conserve U.S. coral reefs. Specifically, Executive Order 13089 directs Federal agencies whose actions may affect U.S. coral reef ecosystems, to (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.

The overarching goal of this SERDP project is to provide quantitative baseline data and develop advanced bio-optical techniques for non-destructive assessment of the viability and health of DoD coral reef communities with the capabilities to identify natural and anthropogenic stressors. The basic hypothesis of this project is that different environmental stressors lead to specific modifications to the coral physiology and are characterized by unique combinations of FIRe fluorescence signatures that can be used for the selective diagnostics of the stressors. Establishing the stress-specific FIRe fluorescence signatures provides the background for development of fluorescence protocols for bio-optical diagnostics of the natural and anthropogenic stresses.

This is an addendum to the SI-1334 Report (Gorbunov and Falkowski 2007). The objectives that were addressed in the first phase of the project and were reported in (Gorbunov and Falkowski 2007) were (1) to develop the novel Fluorescence Induction and Relaxation (FIRe) technique and set of instrumentation, including a bench-top FIRe flurometer and prototype underwater instruments, for non-destructive assessing the physiological status of coral and other benthic organisms; (2) study of the impact of common natural stresses (as elevated temperature and photoinhibition) and selected anthropogenic stressors (copper contamination) on coral; (3) develop algorithms for fluorescence diagnostics of these stresses; and (4) collect baseline data of fluorescence and physiological characteristics of dominant coral species in the Caribbean and Indo-Pacific regions.

Objectives of the second phase of this project that are reported in this addendum are:

- to study the impact of toxic metals, including copper, zinc, lead, and tin on coral photosynthesis and physiology;
- to elucidate the effects of petroleum products on coral;
- to examine the impact of selected bacterial diseases on coral fluorescence and photosynthetic characteristics;
- to study the combined effect of thermal stress and nutrient load on coral fluorescence and photosynthetic characteristics;

- to develop methodology for fluorescence diagnostics of anthropogenic stressors in coral ecosystems;
- to develop a laser-based Fluorescence Induction and Relaxation (FIRe) Sensor for Remotely-Operated Vehicle (ROV) or Diver-Swam Vehicle (DSV) type of platforms.

3. Project Background

The DoD is responsible for numerous facilities in tropical and subtropical environments that are adjacent to coral reefs. Coral reefs are especially susceptible to anthropogenic insult and 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.

This is an Addendum to the SERDP SI-1334 Report (Gorbunov and Falkowski 2007). Within the framework of the first phase of the project (Gorbunov and Falkowski 2007), we developed the novel Fluorescence Induction and Relaxation (FIRe) technique and set of instrumentation for non-destructive assessing the physiological status of coral and other benthic organisms (Gorbunov and Falkowski, 2004). A bench-top version of the FIRe System has been constructed and employed in the laboratory and field research. A prototype laser-based FIRe Sensor has been designed and tested 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. The laboratory studies have established fluorescence signatures of common natural stresses (as elevated temperature and excess light) and selected anthropogenic stresses (such as copper toxicity) 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 (Gorbunov and Falkowski 2007).

With the aim to further develop algorithms for diagnostics of a variety of anthropogenic stressors, we proposed a research program that was funded by SERDP as a second phase of SI-1334 (and then RC-1334) project. The results of this second phase are presented in this Addendum.

Within the framework of the second phase of the project, we have established the cellular mechanisms and fluorescence signatures of the impact of toxic metals, including zinc, lead, cadmium, and tin. Also, we quantified the action mechanisms and fluorescence signatures of other stressors (such as petroleum products) that can be potentially associated with Navy and DoD facilities and might have an adverse affect on coral communities. With the aim to integrate the FIRe technology with the mosaic mapping technique developed within the SI-1333 project (see Reid et al, 2009), we proposed to develop a laser-based FIRe Scanner that might be incorporated into ROV or DSV platforms. This instrumental part of the project follows up the previous development of a laboratory scale model of such a sensor. The strategic goal is to obtain a better understanding of how the developed technology and algorithms can be integrated into a single strategy that can be used by DoD resource managers or their contractors to monitor coral reef health and to assess, when necessary, the impacts of particular stressors.

4. Material and Methods

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 relies on the measurement and analysis of chlorophyll "variable fluorescence" (Fig. 1), a property unique to the photosynthetic machinery (see Falkowski et al, 2004 for review). The variable fluorescence signals are recorded by using Fast Repetition Rate (FRR) Fluorometry (Kolber et al, 1998) or its technological 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 (Table 1).

Fluorescence Induction and Relaxation (FIRe) technique and instrumentation for assessment and monitoring of the physiological status of corals and other benthic photosynthetic organisms - The FIRe technology assesses the physiological status of photosynthetic system in algae and corals rapidly and in a non-invasive way. The FIRe system developed within the framework of this SERDP project allows for a more extensive (compared to previous FRR fluorometers) sampling in the field to be conducted and also provides the basis for development of underwater submersible fluorosensors. Several types of instruments have been developed and used in this research, including a bench-top FIRe fluorometer, a diver-operated fluorometer, and a moorable FIRe fluorometer (Gorbunov and Falkowski 2004) (Fig. 2 and 3). Together with spectral fluorescence and novel molecular biology techniques, FIRe fluorometry provides comprehensive diagnostics of the viability of DoD coral reef ecosystems. 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).

Fluorescence signals from coral samples and zooxanthellae cultures were measured using the custom-built Fluorescence Induction and Relaxation (FIRe) System (Fig. 2). Fluorescence was excited by radiation from blue (450 nm with 30 nm half-bandwidth) light-emitting diodes and recorded in the red spectral region (680 nm with 20 nm bandwidth). The diodes generate microsecond flashes with the peak optical power density up to 1W/cm2 in the sample chamber to ensure fast reduction of PSII reaction centers within a single photosynthetic turnover (from ca. 20 to 80 μs, Fig. 1). Analysis of fluorescence induction on the microsecond time scale provides the minimum (Fo) and maximum (Fm) fluorescence yields, the quantum efficiency of photochemistry in PSII (Fv/Fm), the functional absorption cross-section of PSII (σ_{PSII}), and the rates of photosynthetic electron transport down to carbon fixation (Table 1). Three to five replicates were made for each experiment.

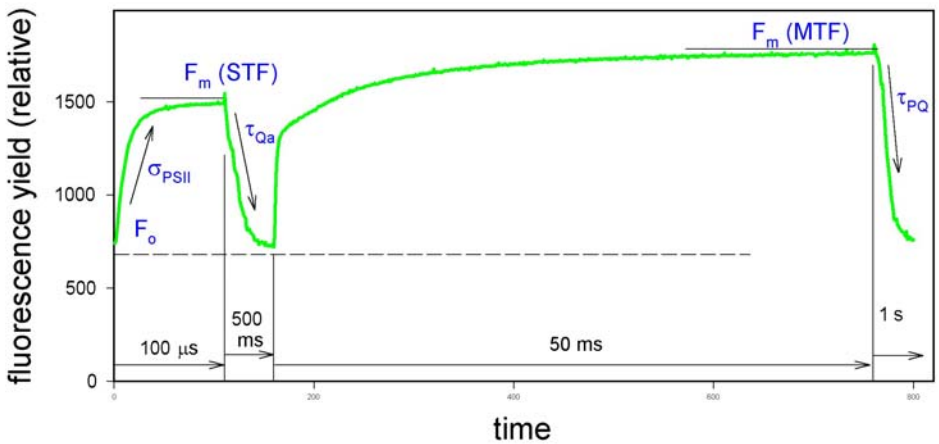

Figure 1. An example of the FIRe fluorescence transients. Kinetics of fluorescence yields is recorded with microsecond time resolution and includes four phases: (1st phase, 100 µs) a strong short pulse of 100 µs duration (called Single Turnover Flash, STF) is applied to cumulatively saturate PSII and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

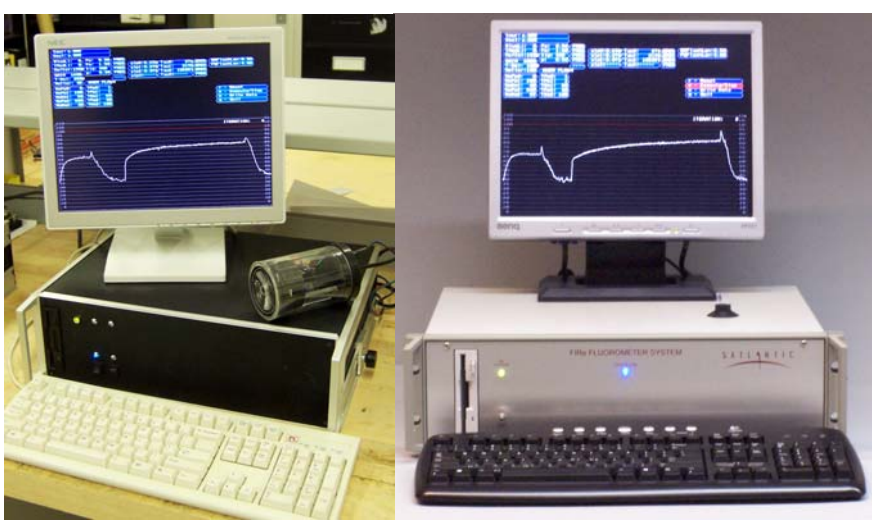

Figure 2. Bench-top versions of the FIRe Fluorometer System. (Left) - FIRe System prototype developed in 2003-2004. (Right) - commercial FIRe Fluorometer System manufactured by Satlantic Inc.

Figure 3. Diver-operated (top) and moored (bottom) FIRe Fluorometer Systems.

Table 1. List of fluorescent and photosynthetic parameters measured with FIRe technique (see Gorbunov and Falkowski, 2004 and Kolber et al 1998 for more detail)

FIRe-retrieved parameter	*Description*
Fo, Fm	Minimum and maximum yields of chlorophyll-a fluorescence measured in a dark-adapted state (arbitrary units). The minimum yield is measured when photosynthetic reaction centers are open; the maximum yield - when the centers are closed.
Fv	Variable fluorescence (= Fm – Fo)
Fv/Fm	Maximum quantum yield of photochemistry in PSII, measured in a dark-adapted state (dimensionless). This parameter characterizes the efficiency of primary photosynthetic reactions. The maximum value of Fv/Fm is ca. 0.55 in healthy corals and usually decreases under stress conditions.
Fo', F', Fm'	Minimum, steady-state, and maximum yields of chlorophyll-a fluorescence measured under ambient light, arbitrary units (the prime character indicates the measurements are made under ambient light)
σ_{PSII}	Functional absorption cross section of PSII (A^2) in a dark-adapted state. The parameter is the product of the optical absorption cross section of PSII (i.e, the physical size of the PSII unit) and the quantum yield of photochemistry in PSII.
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. The parameter characterizes the actual photosynthetic rates achieved under ambient light. ΔF'/Fm' decreases with ambient irradiance compared to Fv/Fm due to saturation of photosynthetic rates under high irradiance.
Fv'/Fm'	Quantum efficiency of photochemistry in open reaction centers of PSII, measured in a light-adapted state (=(Fm'-Fo')/Fm'). This parameter is usually lower than Fv/Fm due to photoinhibition and photosynthetic down-regulation under high light. Both Fv'/Fm' and ΔF'/Fm' are functions of ambient irradiance and photoacclimation history.
p	"Connectivity factor", a parameter that defines the exciton energy transfer between individual photosynthetic units of PSII (dimensionless).
(Fm- Fm') /Fm	Quantum efficiency of non-photochemical quenching (i.e., light-induced thermal dissipation of excess energy under supra-optimal light. This parameter determines the capacity of photoprotective mechanisms under strong light.
τ_{Qa}	Time constant of photosynthetic electron transport on the acceptor side of PSII. The parameter characterizes the rates of Qa re-oxidation, i.e., the photosynthetic electron transfer from Qa to Qb (primary electron acceptor to secondary quinine acceptor of PSII)
τ_{PQ}	Time constant of photosynthetic electron transport between PSII and PSI (i.e., time of plastoquinone pool re-oxidation)

5. Results and Discussion

5.1. Study of the impact of anthropogenic stressors to coral communities and development of bio-optical algorithms for detection and assessment of the stresses

5.1.1. Effect of metal toxicity on coral photosynthesis and fluorescence – Metal contamination is one of the main anthropogenic stressors in coastal ecosystems, including coral communities. The main sources of heavy metals in aquatic environments include anti-fouling paints, sewage, and industrial and agricultural run-off. Identification and quantification of metal poisoning is essential for the development and implementation of the ecosystem protection and remediation.

This research was designed to elucidate physiological mechanisms of heavy metal stress, to establish bio-optical signatures of metal toxicity, and to develop fluorescent methods for recording the response of corals to metals. Controlled experiments have been conducted on laboratory cultures of symbiotic zooxanthellae and the diatom, *Thalassiosira wissflogii*. The zooxanthellae were used as a model for coral behavior, while diatoms as a model for brown algae and benthic macrophytes.

The impacts of five toxic metals, copper, cadmium, zinc, lead, cadmium, and tin have been quantified. The dynamics of the stress development was monitored using FIRe fluorometry that provided insight into alterations to the physiological status of the primary and secondary photosynthetic reactions and the photosynthetic electron transport rates in Photosystem II (Gorbunov et al 2001; Gorbunov and Falkowski 2005).

The research program was designed to test the following basic hypotheses:

(a) Heavy metal exposure leads to a decrease in the quantum efficiency of photosynthetic light utilization (Fv/Fm) in symbiotic zooxanthellae (proven, see results below).

(b) The efficiency of energy transfer within the photosynthetic light-harvesting antennae is compromised under the heavy metal stress, leading to decline in the energy transfer between photosynthetic units (the "connectivity factor" which characterizes the integrity of photosynthetic complexes) and the cross section of Photosystem II (i.e., the functional size of the Photosystem II antenna) (proven);

(c) The disruption of the energy transfer in primary photosynthetic reactions stimulates accumulation of triplet states in light-harvesting complexes that will be evident from the triplet quenching of chlorophyll fluorescence and the enhanced ROS production (proven);

(d) The rates of electron transport in Photosystem II and between PSII and PSI may be reduced under the impact of metal toxicity (proven - under conditions of high irradiance; however, the rates were not affected in low-light acclimated organisms);

(e) In intact coral, heavy metal stress affects both the coral host and the photosynthetic symbionts, with stronger impact on the host. Metal exposure would enhance caspase activity in the coral tissue (proven, thereby the impact of metal toxicity on zooxanthellae was stronger under high-light conditions);

(f) Metal toxicity will be higher for copper, cadmium, and lead and lower for zinc and tin (proven);

(g) The biophysical and physiological signatures of heavy metal stress differ from those of natural stresses, such as temperature and access light stresses (proven).

5.1.1.1. Thresholds of metal toxicity - To assess the thresholds of metal toxicity, we exposed the cultures of zooxanthellae and diatoms to a range of metal concentrations from non-toxic to highly toxic levels (e.g., 1, 5, 10, 25, 50, 100, 200 μM for Cu, Cd, and Zn; and 5, 25, 100, 200, 400 μM for Pb and Sn). For each treatment, we monitored detrimental change in the growth rates and photosynthetic efficiency (Fv/Fm) over the period of ~1 week. Based on these observations, we have determined the thresholds of metal toxicity and the concentration of each metal that elicited an adverse effect within two to three days of metal exposure (i.e., within the average lifetime of the cells in the population). The following metal concentrations were chosen for further detailed examination: 50 μM for Cu, 25 μM for Cd, 100 μM for Zn, 100 μM for Pb, and 200 μM for Sn.

Because the metal toxicity effects in plants and the sites of action may vary with growth irradiance, we conducted laboratory experiments for the cells grown under low and high light (50 and 300 μM quanta cm^{-2} m^{-1}, respectively). These experiments are important to understand the signatures of metal toxicity in both shallow and deep benthic ecosystems. Below we present examples of metal-induced alterations in fluorescent and photosynthetic characteristics and discuss FIRe fluorescent signatures of metal toxicity.

5.1.1.2. Cadmium toxicity – Fig. 4 shows the effects of cadmium toxicity on the FIRe photosynthetic parameters of zooxanthellae. The analysis revealed that the time of electron transport between the photosystems (tau-mtf) was the first parameter which responded to Cd poisoning. This parameter increased within a day and remained high throughout the experiment. The next inhibited parameter was the maximum rate of photosynthesis (Pmax). The "connectivity factor" (p) decreased significantly after 2-3 days. All three parameters were altered prior to any changes in the quantum efficiency of photochemistry in PSII (Fv/Fm) or the rate of electron transport in PSII (tau-stf) occurred. The effective cross-section of PSII (σ_{PSII}) remained unaltered within the measurement precision of approximately 10% (data not shown). The temporal evolution of the photosynthetic characteristics suggests that the Cd poisoning first affects the integrity of photosynthetic lipid membranes and inhibits the secondary photosynthetic reactions, whereas the primary photosynthetic reactions are affected at later stages of stress development.

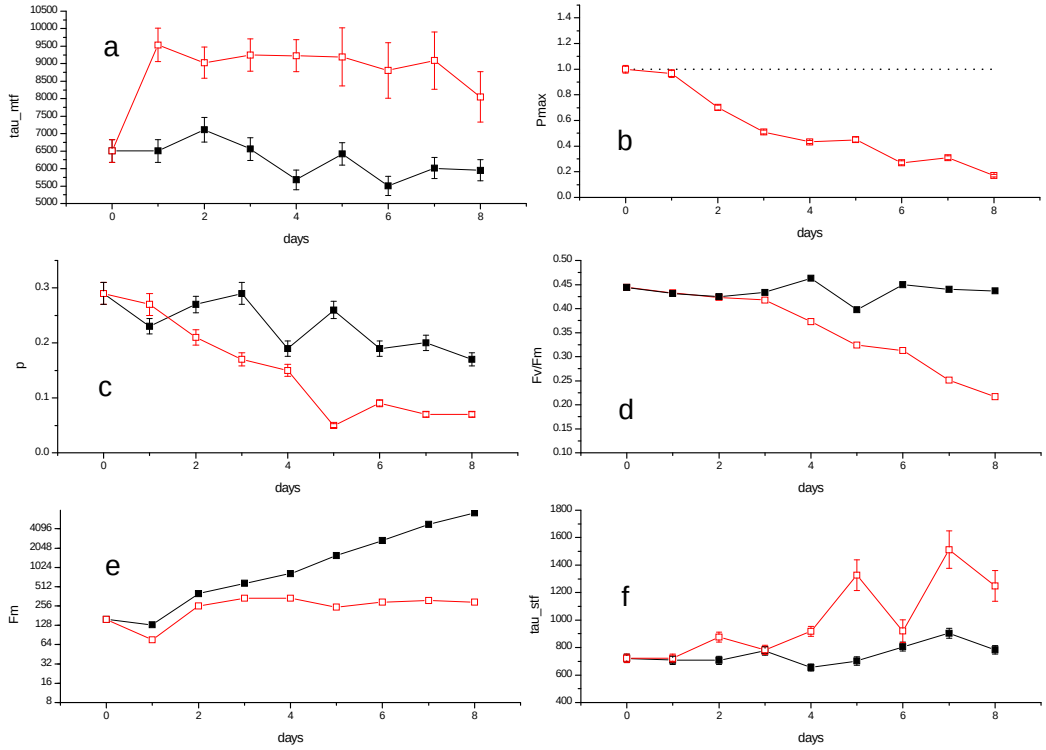

Figure 4. Effect of Cd (25 μM) toxicity on the zooxanthellae, *Symbiodinium sp. CCPM 2476*. Temporal evolution of photosynthetic parameters including the rates of photosynthetic electron transport between PSII and PSI (tau-mtf , microseconds, panel **a**), the maximum photosynthetic rate (Pmax, panel **b**), the energy transfer in the light-harvesting antennae ("connectivity factor" p, panel **c**), the quantum efficiency of photochemistry in PSII (Fv/Fm, panel **d**), maximum yield of chlorophyll fluorescence (Fm, panel **e**), and the rate of electron transport on the acceptor side of PSII (tau-stf, microseconds, panel **f**). Open dots show changes under Cadmium treatment, filled dots - Control, High Light treatment. The maximum rates of photosynthesis (Pmax, panel **b**) are normalized to the values of Pmax in the control.

5.1.1.3. Copper toxicity – Fig. 5 shows an example of the temporal evolution of FIRe photosynthetic parameters under Cu poisoning. Similar to Cd, Cu poisoning starts with the impairment of the rate of electron transport between the photosystems (tau-mtf); however, this parameter recovers after approximately 2 days. The "connectivity factor" is affected at the same time as tau-mtf (Day 1-2). The response in Pmax is delayed and matches that in Fv/Fm and the inhibition of the growth rates (as evident from the alteration in Fm, a proxy of algal growth). The temporal evolution pattern, therefore, suggests co-inhibition of both primary and secondary photosynthetic reactions by Cu. Thereby, the high-light conditions enhanced the decrease in Pmax (as compared to Fv/Fm), suggesting a stronger inhibition of secondary photosynthetic reactions. The fluorescence signatures of Cu toxicity in diatoms were similar to those in zooxanthellae, implying common physiological effects on the photosynthetic reactions.

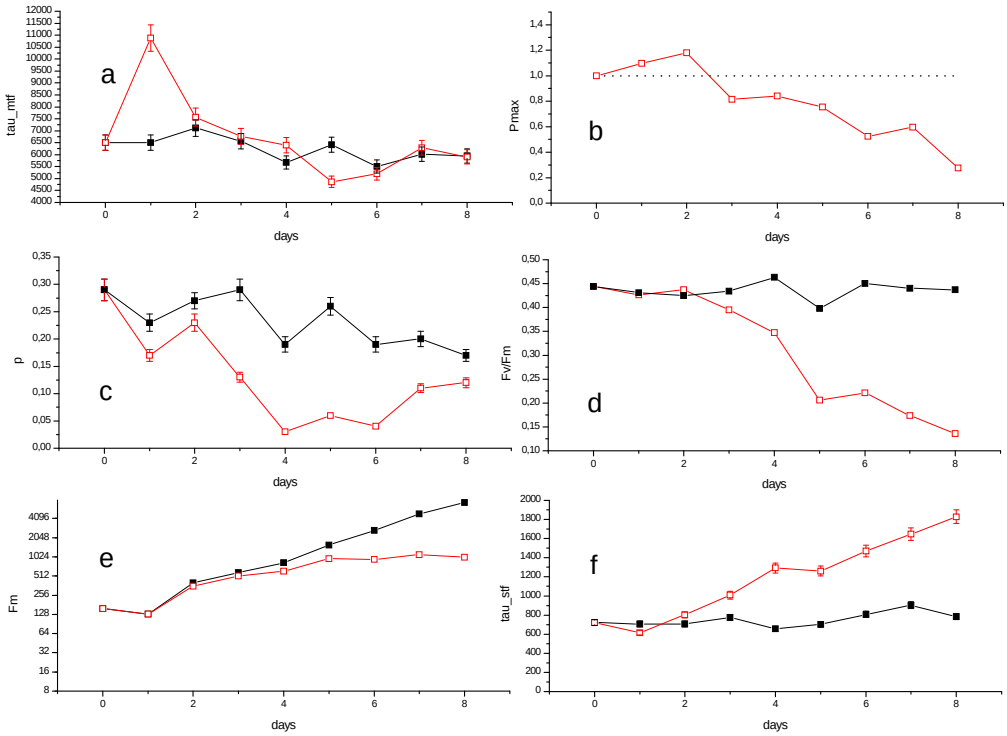

Figure 5. Effect of Cu (50 µM) poisoning on the zooxanthellae, *Symbiodinium sp. CCPM 2476*. Temporal evolution of photosynthetic parameters including the rates of photosynthetic electron transport between PSII and PSI (tau-mtf , microseconds, panel **a**), the maximum photosynthetic rate (Pmax, panel **b**), the energy transfer in the light-harvesting antennae ("connectivity factor" p, panel **c**), the quantum efficiency of photochemistry in PSII (Fv/Fm, panel **d**), maximum yield of chlorophyll fluorescence (Fm, panel **e**), and the rate of electron transport on the acceptor side of PSII (tau-stf, microseconds, panel **f**). Open dots – copper treatment under high light growth; filled dots - for control.

5.1.1.4. Zinc toxicity – Similar to Cd and Cu, Zn poisoning started with impairment of the rate of electron transport between the photosystems (tau-mtf) (Fig. 6a), although the change in this parameter was less pronounced. Changes in the time of electron transfer on the acceptor side of PSII (i.e. the rate of Qa re-oxidation, Fig. 6f) and the connectivity factor (Fig. 6c) occurred earlier than that in Fv/Fm (Fig. 6c) or Pmax (Fig. 6b). This pattern of temporal evolution may indicate that lipids are more susceptible to Zn poisoning than the proteins of PSII complex. The detrimental changes in the above photosynthetic characteristics followed the decline in the cell growth rate, as evident from delayed response in Fm, a proxy of chlorophyll biomass (Fig. 6e).

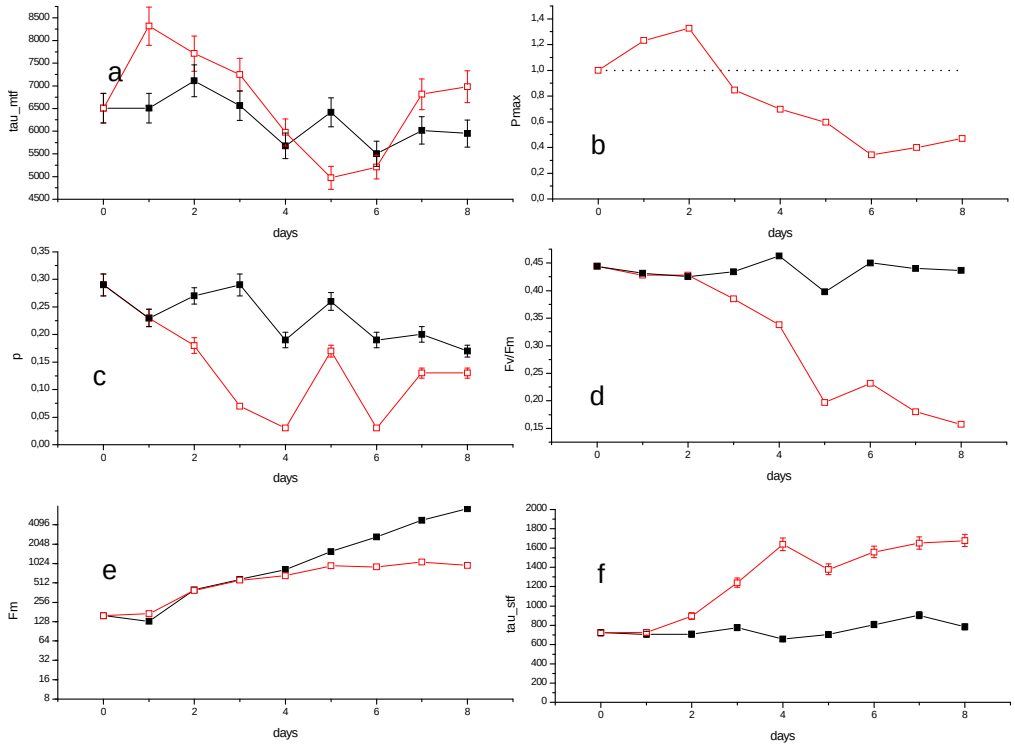

Figure 6. Effect of Zn (100 μM) poisoning on the zooxanthellae, *Symbiodinium sp. CCPM 2476*. Temporal evolution of photosynthetic parameters including the rates of photosynthetic electron transport between PSII and PSI (tau-mtf , microseconds, panel **a**), the maximum photosynthetic rate (Pmax, panel **b**), the energy transfer in the light-harvesting antennae ("connectivity factor" p, panel **c**), the quantum efficiency of photochemistry in PSII (Fv/Fm, panel **d**), maximum yield of chlorophyll fluorescence (Fm, panel **e**), and the rate of electron transport on the acceptor side of PSII (tau-stf, microseconds, panel **f**). Filled dots - for Control, High Light treatment; open dots – Zn treatment.

5.1.1.5. Lead toxicity - Similar to other metals, Pb poisoning starts with a characteristic change in the rate of electron transport between the Photosystem II and Photosystem I (tau-mtf, Fig 7a). However, in contrast to other metals, the "connectivity factor" remained unaltered, even after growth rates, Pmax, and Fv/Fm were impaired. The effective cross-section of PSII and the rate of electron transport in PSII (tau-stf) also remained unaffected. The growth rates and, hence, the cell division were inhibited stronger and faster by lead than the efficiency of the photosynthetic reactions. Also, the inhibition of the growth was virtually coincident with the impairment in the quantum efficiency of photochemistry in PSII (Fv/Fm) and the maximum rate of photosynthesis Pmax. This pattern suggests the simultaneous effects of Pb toxicity on multiple sites in both photosynthetic reactions and cellular metabolism. Note that in contrast to other metals, Pb affects the photosynthetic efficiency (Fv/Fm) only under severe poisoning.

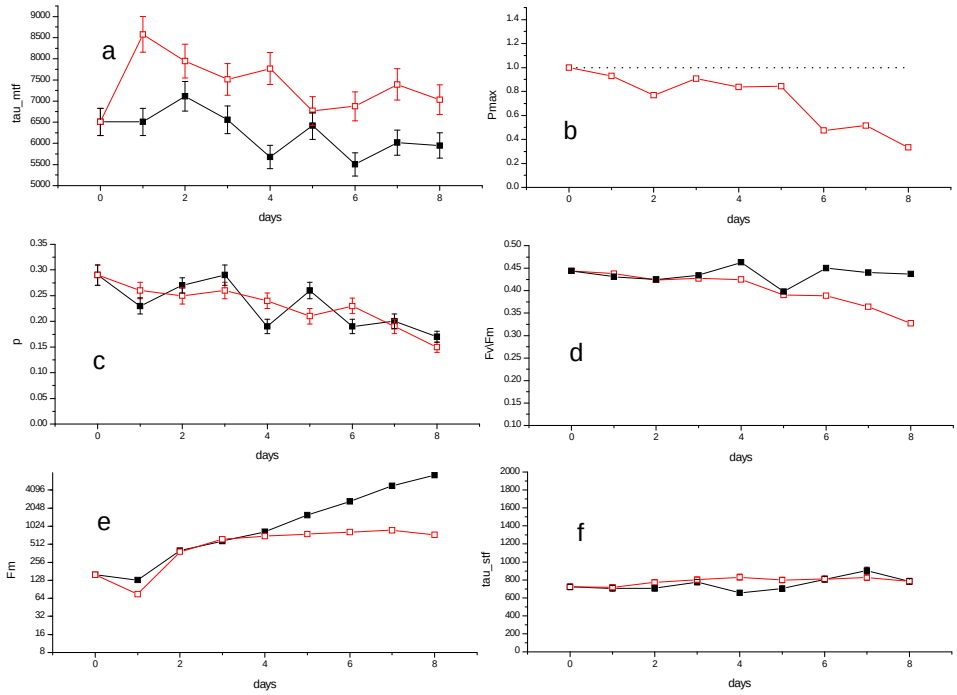

Figure 7. Effect of Pb (100 μM) poisoning on the zooxanthellae, *Symbiodinium sp. CCPM 2476*. Temporal evolution of photosynthetic parameters including the rates of photosynthetic electron transport between PSII and PSI (tau-mtf , microseconds, panel **a**), the maximum photosynthetic rate (Pmax, panel **b**), the energy transfer in the light-harvesting antennae ("connectivity factor" p, panel **c**), the quantum efficiency of photochemistry in PSII (Fv/Fm, panel **d**), maximum yield of chlorophyll fluorescence (Fm, panel **e**), and the rate of electron transport on the acceptor side of PSII (tau-stf, microseconds, panel **f**). Open dots – Pb treatment under high light growth conditions; filled dots - control.

5.1.1.6. Tin toxicity – Sn poisoning led to a marked decrease in the maximum rate of photosynthesis (Pmax) observed at the same time as impairment of growth rate. However, tin had only minor (~ 15% decline) impact on the photosynthetic efficiency of PSII (Fv/Fm) and no effect on the rates of photosynthetic electron transport in PSII and between PSII and PSI (see Johnson-Worrell and Gorbunov, 2011 for details). These results clearly suggest that tin exposure impairs the secondary photosynthetic reactions, with virtually no impact of the primary photosynthetic reactions in PSII. Clearly, the FIRe fluorescence signatures of tin toxicity differ strikingly from those of other metals (as Cd, Cu, or Zn).

5.1.1.7. Effect of growth irradiance on the physiological response to metal toxicity – The effects of heavy metal ions and the sites of action may vary with environmental conditions, such

as growth irradiance [Kuepper 2002]. Our FIRe fluorescence analysis further revealed that the alterations in the photosynthetic and fluorescence characteristics differ in the cultures grown under low (LL) and high light (HL). The analysis revealed that:

1. In zooxanthelae and diatoms acclimated to HL, poisoning with Cu, Zn, or Cd leads to a characteristic increase in the time constant for the photosynthetic electron transport rate between PSII and PSI (e.g., Fig. 5a). This variable is the first signature of metal toxicity in HL-acclimated cells. However, when the cells are grown under LL, the rate of electron transport between PSII and PSI remains unaltered (within approximately 10-15%) by the metals. Further analysis revealed that the rate of electron transport between PSII and PSI was already reduced under LL acclimation (due to physiological photoacclimation) and metal exposure did not affect it any further.
2. High-light environment accelerates the development of metal toxicity (for all metals, except Pb);
3. In the high-light environment, the metal induced reduction in the maximum rates of photosynthesis (Pmax) was faster and more dramatic. The decrease in Pmax is a marked signature of metal toxicity.
4. The above signatures of metal toxicity are most pronounced under high light and, thus, are the most efficient diagnostic markers on shallow reefs.

Therefore, the FIRe fluorescent assessment of metal toxicity in coral zooxanthellae revealed that metal poisoning exhibit specific fluorescence and photosynthetic signatures that differ from the signatures of common natural stressors, such as thermal stress, photo-inhibition, or nutrient load. Also, the fluorescent signatures and, hence, the physiological mechanisms of metal toxicity differ between the metals examined. The difference in FIRe fluorescence signatures was most pronounced between essential (as Cu and Zn) and non-essential metals (as Pb). These results provided a biophysical background for development of FIRe fluorescence protocols for diagnostics of the metal toxicity in coral and other benthic photosynthetic organisms.

5.1.2. Effect of petroleum products on coral photosynthesis

Petroleum-based products are a complex mixture of multiple chemical compounds, including a variety of hydrocarbons that may exhibit different toxicity. Some of the chemicals (benzene, toluene, naphthalene, xylenes, polycyclic aromatic hydrocarbons PAHs, heavy metals, etc.) are significant because of their toxicity or smell.

The objective of this project was to characterize the biophysical and physiological mechanisms of petroleum toxicity in corals, to identify the set of fluorescent parameters that can be potentially used as a diagnostic signature of petroleum toxicity. We have examined the toxicity of selected individual petrochemicals, including benzene, hexane, pentene, nonane, decane, as well as complex mixtures such as gasoline and kerosene. Basic experiments have been conducted on cultures of zooxanthellae isolated from corals.

Our previous investigation of natural stressors (elevated temperature, photoinhibition, and nutrient load) and metal toxicity revealed that these stressors lead to gradual, long-term detrimental change in photosynthetic characteristics (Gorbunov and Falkowski 2007). For

example, the exposure to sub-lethal concentrations of heavy metals becomes detectable only after 12 to 48 hours. In contrast, the impact of petroleum is characterized by both short-term (minutes to hour) dynamic response and subsequent long-term (days) impairment of photosynthesis.

5.1.2.1. Short-term response to petroleum poisoning –

The **s**hort-term response of photosynthetic characteristics of zooxanthellae to benzene is shown in Fig. 8. The fluorescence analysis revealed that the petrochemical poisoning starts with an immediate decrease in the functional cross-section of Photosystem II (σ_{PSII}) and the efficiency of energy transfer between PSII units (p), followed by a slower (~ 10 minutes) reduction in the efficiency of photochemistry in PSII (Fv/Fm) and a dramatic reduction in the rates of electron transport on the acceptor side of PSII (Qa re-oxidation). It should be noted that the exposure to 0.05 g/L does not affect the photosynthetic efficiency of PSII (Fv/Fm), but leads to dramatic change in other photosynthetic parameters, including the rates of electron transport on the acceptor side of PSII and parameter *p*. The use of FIRe multi-parameter protocols allows the detection of petroleum toxicity at concentrations as low as ~ 30 ppm (see Table 2).

Fig. 9, 10, and 11 show examples of the **s**hort-term responses of photosynthetic characteristics to hexane, nonane, and jet fuel, respectively. The exposure to most petroleum products (except of heavy alkane hydrocarbons, such as n-decane or n-dodecane) leads to an immediate (within < 10 s) decrease in the functional cross-section of Photosystem II and the efficiency of energy transfer between PSII units (Fig. 8-10). The change in the latter parameter is dramatic (by a factor of ~10) and was not previously observed under any other stressors. The initial fast response is followed by a slower (~ 10 minutes) reduction in the efficiency of photochemistry in PSII (Fv/Fm) and a dramatic reduction in the rates of electron transport on the acceptor side of PSII (Qa re-oxidation). The fast response suggests rapid absorption of the chemicals into the cell and impact on thylakoid lipid membranes (the primary target of the stressor), followed by slower penetration of petroleum into the protein core complexes of Photosystem II. After the initial detrimental change, all the photosynthetic parameters recover slowly (within an hour), suggesting slowly established equilibrium in petroleum concentration across the lipid membranes and balance in the ionic force. While the recovery in some parameters (such as the rates of electron transport) is complete within 20 to 30 minutes, the recovery in others (the efficiency of photochemistry and the cross-section of PSII) takes longer and is partial at moderate and high concentrations of petroleum.

The parameter Fv/Fm is most commonly used in plant physiology and photosynthesis research as a stress indicator. Surprisingly, our research showed that Fv/Fm appears to be the least sensitive to sublethal amounts of petrochemicals (Fig 8, 9, 10, and Table 2). For instance, under the exposure of 0.08 g/L of benzene, Fv/Fm did not change (Fig 8, blue line in the upper panel), whereas t_{Qa} increased dramatically – by a factor of 3 (Fig. 8, lower panel). The above FIRe-derived signatures and responses are unique for petroleum toxicity and were not observed under any other known stress, including nutrient limitation, thermal stress, photoinhibition, or heavy metal toxicity (Gorbunov 2007). The discovered short-term response of the photosynthetic machinery to petroleum exposure provides the basis for development of rapid bioassay protocols for assessment of petroleum exposure in photosynthetic organisms.

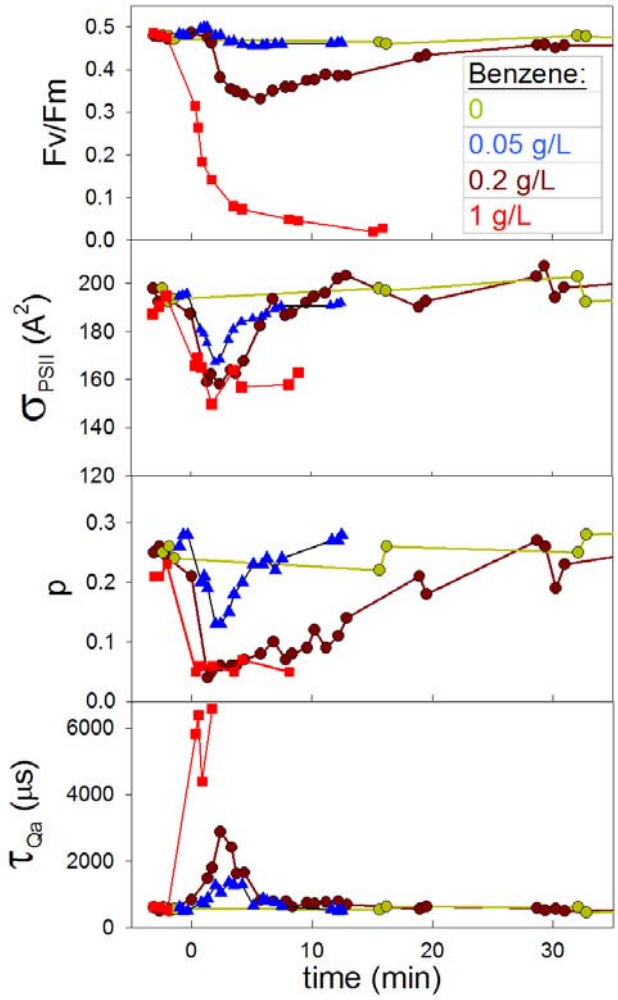

Figure 8. Short-term response of photosynthetic characteristics of coral zooxanthellae to benzene (added at time zero). The temporal evolution of the efficiency of photochemistry in PSII (Fv/Fm); the functional cross-section of PSII (σ_{PSII}); the efficiency of energy transfer between PSII units (p); and the rate of photosynthetic electron transport on the acceptor side of PSII (Qa re-oxidation).

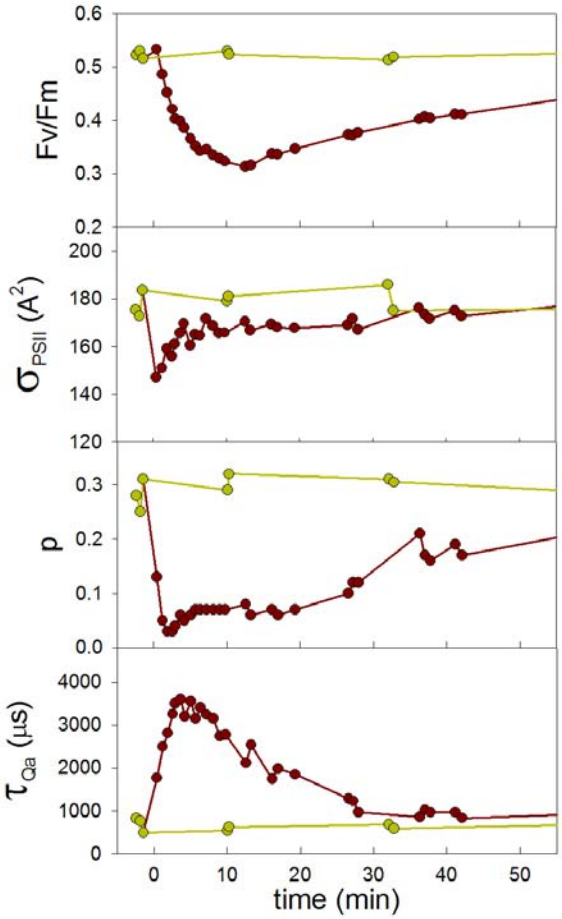

Figure 9. Kinetics of the short-term response of photosynthetic characteristics of coral zooxanthellae to hexane (0.4 g/L added at time zero). The temporal evolution of the efficiency of photochemistry in PSII (Fv/Fm); the functional cross-section of PSII (σ_{PSII}); the efficiency of energy transfer between PSII units (p); and the rate of photosynthetic electron transport on the acceptor side of PSII (Qa re-oxidation).

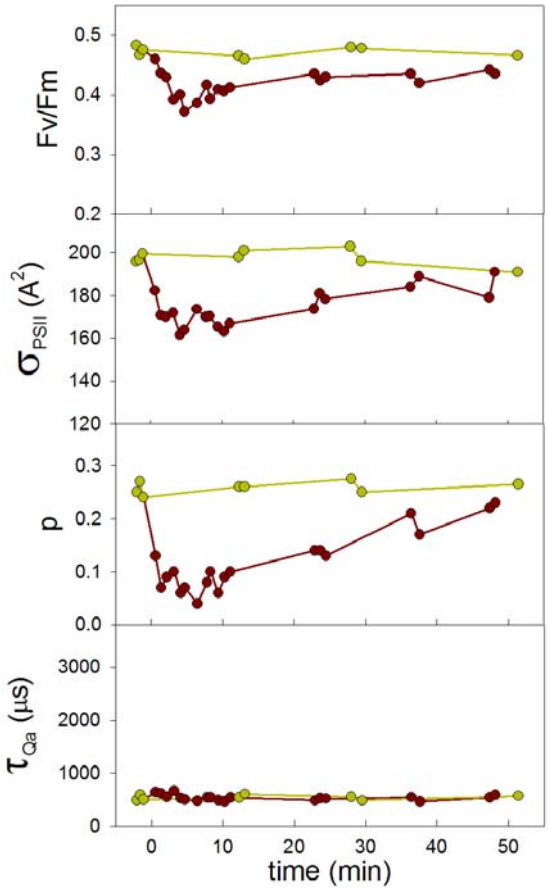

Figure 10. Kinetics of the short-term response of photosynthetic characteristics of coral zooxanthellae to heavier hydrocarbons (n-nonane, 0.8 g/L added at time zero). Temporal evolution of the efficiency of photochemistry in PSII (Fv/Fm); the functional cross-section of PSII (σ_{PSII}); the efficiency of energy transfer between PSII units (p); and the rate of photosynthetic electron transport on the acceptor side of PSII (Qa re-oxidation).

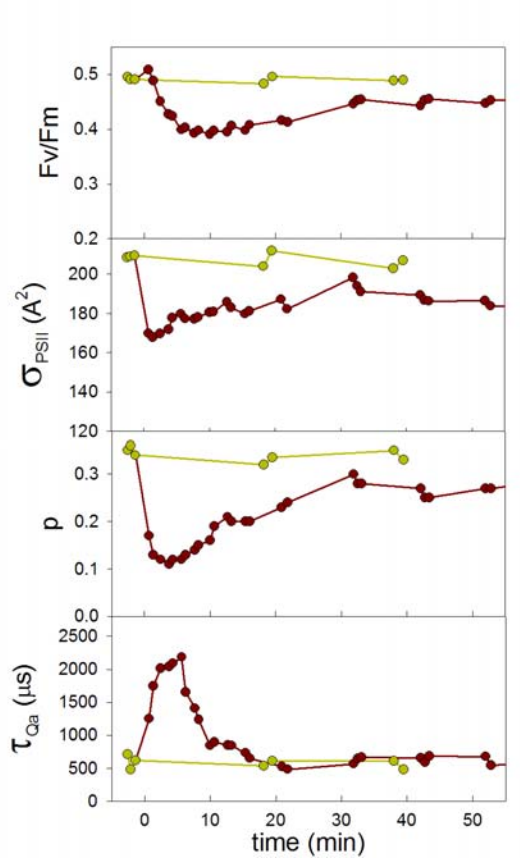

Figure 11. Kinetics of the short-term response of photosynthetic characteristics of coral zooxanthellae to jet fuel (0.5 g/L added at time zero). Temporal evolution of the efficiency of photochemistry in PSII (Fv/Fm); the functional cross-section of PSII (σ_{PSII}); the efficiency of energy transfer between PSII units (p); and the rate of photosynthetic electron transport on the acceptor side of PSII (Qa re-oxidation).

Table 2. Detectivity limits of the FIRe fluorescence bioassay protocols for rapid assessment of petroleum toxicity by using symbiotic dynoflagellates, *Symbiodinium sp*. as a bioindicator.

Petroleum product	Fv/Fm protocol	Extended FIRe protocol
Gasoline 87 AKI	300 ppm	30 ppm
Kerosene	200 ppm	15 ppm
Benzene	300 ppm	20 ppm
Crude Oil, Louisiana Light	400-500 ppm	30 ppm

The Fv/Fm protocol relies on recording a detrimental change in the photosynthetic efficiency of Photosystem II (Fv/Fm) by the value of the measurement uncertainty. Fv/Fm is the most common photosynthetic parameter recorded by many commercial fluorometers (e.g., PAM fluorometers). Clearly, the Fv/Fm parameter is not sensitive enough to sublethal amounts of

petrochemicals. The drastic improvement in the detectivity levels of our proposed FIRe bioassay protocols is achieved by the analysis of the FIRe parameters most sensitive to petroleum insult. These parameters include the energy transfer in light-harvesting antennae (connectivity parameter, p), the functional cross-section of PSII, and the rates of electron transport on the acceptor side of PSII (see Fig 8).

5.1.2.2. Long-term response – The long-term detrimental alterations in the cell are developed on the time scales of days and include gradual impairment of cell growth and photosynthetic reactions. Such response was evident from alterations in variable fluorescence signatures, including a characteristic decrease in the efficiency of primary photochemical reaction in Photosystem II (Fv/Fm parameter), modification in the light-harvesting antennae of PSII, reduction in the rates of photosynthetic electron transport in Photosystem II and in the transport between PSII and PSI (Fig. 12). Although the above signatures were previously established in other stressors, the long-term impact of petroleum proceeds without reduction in the maximum photosynthetic rates that is in striking contrast to heavy metal toxicity.

The long-term fate of the organism contaminated with petroleum correlated with the magnitude of the short-term response. As such, the short-term FIRe signatures can be used for prognosis of longer term toxicity effects.

Overall, the results suggest that, although the fluorescent signatures of petroleum poisoning overlaps with the signatures to other stressors, the physiological mechanisms of this stressor are unique and the use of multi-parameter fluorescent protocols provides a good potential for selective identification of the stressor.

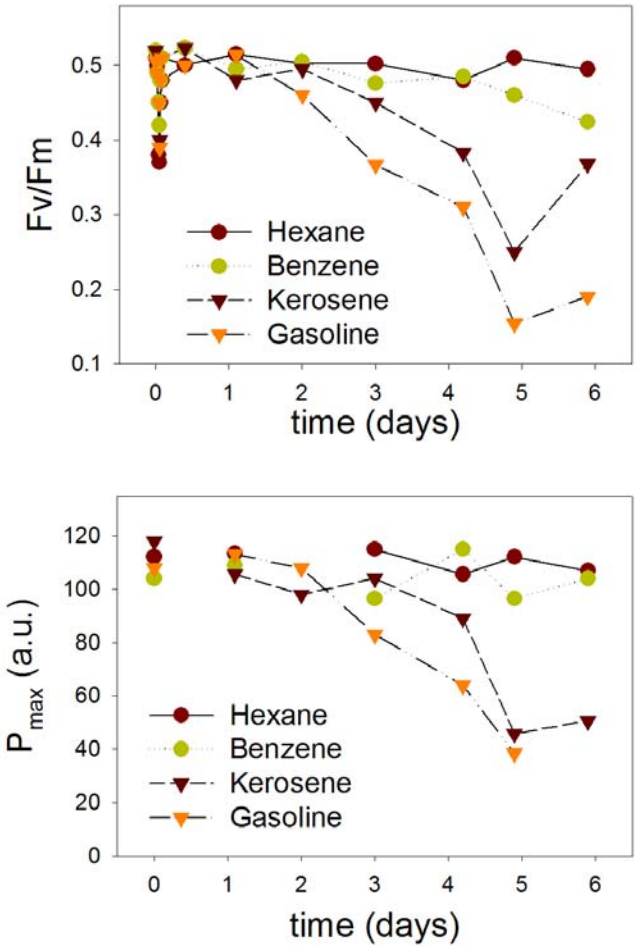

Figure 12. Example of long-term alterations in the photosynthetic efficiency of Photosystem II (Fv/Fm) and the maximum rate of photosynthesis (Pmax) under exposure to petrochemicals. The long-term toxicity varies markedly with petroleum products, but is characterized with a parallel decline in Fv/Fm and Pmax. The concentrations of petrochemicals in water were 0.3 g/L for hexane, 0.2 g/L for benzene, 0.5 g/L for kerosene, and 0.5 g/L for gasoline 87 AKI.

5.2. <u>Effect of multiple stressors on coral photosynthesis and physiology</u>

As a model of multiple stressors, we examined the combined effect of nutrient load and thermal stress. The individual effects of these stressors have been studied previously (Tchernov et al, 2004; Gorbunov and Falkowski, 2007). When acting individually, these two stressors show opposite effects on the photosynthetic efficiency (F_v/F_m) of coral – the nutrient load leads to an increase in F_v/F_m, whereas the thermal stress decreases F_v/F_m. It was expected that the combined effects of the two stressors could produce no change in the measured signals, resulting in failure of diagnostic protocols under certain conditions. Here, we specifically analyzed how the FIRe

fluorescence parameters (other than F_v/F_m) are affected by the two stresses with the goal to identify the most appropriate set of parameters and to develop a protocol that would allow us to avoid or minimize the risk of failure of fluorescence diagnostics. We further evaluated under what conditions the fluorescence diagnostics may work or not.

The experiments were conducted on zooxanthellae symbionts (*Symbiodinium microadriaticum CCMP2467* and *CCMP832*) isolated from coral and grown in laboratory cultures under nutrient-replete and nitrogen-deplete conditions (Fig 13). The nutrient-replete culture was used as a model for corals under nutrient load, whereas the second as a model for healthy corals in clear, nutrient-poor tropical waters. The extent of nutrient limitation was monitored by the Fv/Fm parameter that was 0.6-0.62 in nutrient replete cultures and decreased to 0.45-0.50 under nutrient limitation. The Fv/Fm values of ca. 0.50 are indicative of moderate nutrient limitation and are typical for corals in clear tropical waters (Gorbunov et al, 2000). Samples from both cultures were then exposed to thermal stress (30 and 32 degree C, with 26 degree C in the control). The evolution of thermal stress development and recovery has been monitored over the period of two to three weeks in each experiment (Fig 13).

The research revealed that both nutrient-rich and deplete cultures responded to thermal stress in a similar way - by a characteristic decrease in Fv/Fm (down to 0.2 under severe stress). Thereby, the change induced by thermal stress was significantly stronger than the difference between nutrient-rich and deplete conditions. Also, the thermal exposure led to a characteristic reduction in the rates of the photosynthetic electron transport on the acceptor side of PSII. This is the second signature of the thermal stress in coral. Fig. 13 (lower panel) shows the evolution of the time constant (i.e., the reciprocal rate) of the electron transport in PSII. Although nutrient load alone did not affect this time constant t_{Qa} (as evident from the readings at time zero), the thermal stress led to an increase in t_{Qa} in both nutrient-replete and N-limited cultures. Therefore, the increase in the time constant t_{Qa} is indicative of the thermal stress development in both control and nutrient enriched environment and can be readily used together with Fv/Fm as a fluorescent signature of thermal stress. The diagnostics of thermal stress was found to be challenging only in the case of minor thermal stress superimposed on significant nutrient loading. In this case, nutrient-induced increase in Fv/Fm appeared to be compensated by thermal-induced decrease in Fv/Fm. However, the impact of thermal stress was minor in this case and the detrimental change showed fast recovery when the normal temperature was restored (physiologically speaking, thermal impact has not yet developed into stress). Also, because nutrient loading leads to significant increase in coral pigmentation that remains unaltered at early stages of thermal stress development, this signature of nutrient loading was readily detectable even when superimposed on a moderate thermal impact. Therefore, our research clearly suggests that FIRe fluorescence diagnostics are capable of identifying and quantifying the thermal stress, even when it is superimposed on nutrient loading.

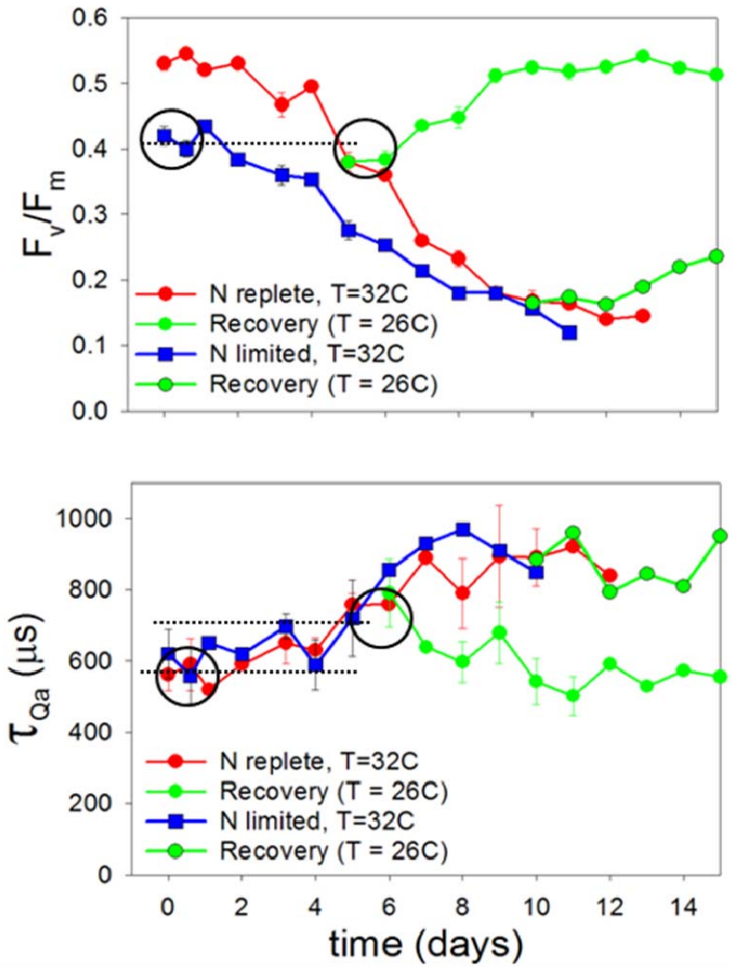

Figure 13. Combined effect of nutrient load and thermal stress on photosynthetic characteristics of the zooxanthellae, *Symbiodimium microadriticum CCMP2467*. (**Upper panel**) - Temporal evolution of the quantum efficiency of photochemistry in PSII (Fv/Fm) in nutrient-replete (red) and nitrogen-limited (blue) cultures exposed to thermal stress. Green dots show the recovery in photosynthetic activity when the normal temperature (26 C) was restored at t=5 days and 10 days, respectively. (**Lower panel**) - Temporal evolution of the time constant for the electron transport on the acceptor side of PSII (τ_{Qa}).

5.3. <u>Effect of bacterial infection on photosynthesis and fluorescence characteristics of zooxanthellae</u>

The effects of coral diseases on fluorescence and photosynthetic signatures have been studied using the example of Yellow Band Disease (YBD). This coral disease is caused by infection with the bacteria, *Vibrio spp*.. Field observations revealed that YBD is one of the most common diseases in corals, with up to ~ 30% of coral communities being affected in coastal areas during

outbreaks of YBD. The literature data suggest that the primary target of YBD is zooxanthellae, although the Cnidarian host may also be affected at later stages. YBD is accompanied by paling of coral coloration and by a decrease in photosynthetic efficiency that resembles coral bleaching. For instance, early reports referred to *Vibrio* infection in coral as "bacterial bleaching". We examined the physiological mechanisms and fluorescence signatures of YBD using laboratory cultures of isolated zooxanthellae that were inoculated with *Vibrio spp*. bacteria. Also, the fluorescence signatures of *Vibrio* attack on zooxanthellae have been compared with the signatures of intact corals infected with YBD.

With the goal of understanding the role of bacterial infection in the development of coral diseases, we first conducted a series of controlled laboratory experiments on the impact of the bacteria, *Vibrio spp* on isolated zooxanthellae. A variety of *Symbiodinium spp.* zooxanthellale (clades A, B, C, and D) were inoculated with shell-fish and coral-disease bacteria, *Vibrio spp*., isolated from coral yellow-band disease. In each experiment, the bacterial stress development was monitored over the period of one week using FIRe fluorometry and standard biochemical techniques. The analysis revealed that all coral symbionts showed a decrease in cell cycle mitotic index, reduction in pigment content (a sign similar to coral bleaching), cell degeneration and lysis. The exposure to *Vibrio* was accompanied by a marked reduction (by 20-40%, $p < 0.05$) in the rates of photosynthetic electron transport in Photosystem II. However, the efficiency of photochemical conversion in PSII (Fv/Fm) remained unaltered (within ~ 2-3% precision of the measurements). The results suggest that bacterial attack may compromise the function and integrity of the thylakoid membranes, with no impact on the structure of Photosystem II complexes. This pattern is in striking contrast to that of thermal stress that was previously found to start with impairment of Photosystem II and a decrease in Fv/Fm. The toxicity during *Vibrio* stress was highest in clades C1 and B1. Therefore, the sensitivity to bacterial infection varied markedly within a single clade. Surprisingly, both bacteria-sensitive and resilient strains of zooxanthellae were found among each clade. Also, the resilience to bacterial attack did not correlated with the thermal sensitivity of the clades. For example, the analysis revealed that the most thermally sensitive culture of *Symbiodinium microadriaticum, CCMP832,* was the least sensitive to bacterial infection. These results suggest that the ecological pattern of YBD distribution in natural coral communities may differ substantially from those of thermal bleaching. This research helps us to uncover the cellular impairment mechanisms associated with pathogen stress and develop operational protocols for stress diagnostics.

The effects of Yellow Band Disease (YBD) on fluorescence signatures of intact corals were examined on the Caribbean coral, *Montastraea faveolata*, during an outbreak of YBD in the Florida Keys in June 2010. The field measurements were conducted on coral reefs off the Mote Marine Research Lab. The analysis confirmed that the FIRe fluorescence technology was readily capable of detecting a detrimental change in the photosynthetic activity in YBD-infected corals. As in the laboratory cultures of zooxanthellae, YBD in coral led to a marked decrease (by 20-50%) in the rates of photosynthetic electron transport in Photosystem II. However, in contrast to isolated zooxanthellae, the efficiency of photochemical conversion in PSII (Fv/Fm) was also reduced (by 30-40%) compared to healthy corals. Because the outbreak of YBD was observed on shallow reefs, the observed reduction in Fv/Fm may be caused by the combined effect of bacterial attack and high light stress (photo-inhibition). This conclusion is further supported by visual observations of YBD-infected coral on shallow, but not deep coral communities of the same reef.

5.4. Development of Prototype FIRe Fluorosensor for Remotely-Operated Vehicles or Diver-Swam Vehicles (ROV/DSV)

We have previously evaluated the potential of FIRe fluorescence measurements from a ROV platform and designed a laboratory-scale model of the FIRe Sensor for such a platform (Gorbunov, 2007). The main challenge in developing the ROV/AOV type of instrument is the need to increase the operational distance. The feasibility studies at Rutgers Coral Lab revealed that the ROV FIRe sensor can perform measurements at a distance of up to 2 to 3 m (compared to 5 to 10 cm in the diver-operated FIRe instrument) in clear tropical waters. The improvement in the sounding distance has been achieved by incorporating a diode laser excitation source into the FIRe system and improving the fluorescence collection efficiency.

The technical objective of this project was to develop and iteratively improve an underwater FIRe instrumental package for a ROV/DSV platform. This task included design and development of (1) the optical module; (2) electronic package; (3) underwater housing and mechanical components; (4) and software package to control the measurement protocols, data collection, and analysis.

5.4.1. Optical design - The FIRe Sensor (Fig . 14) employs a pulsed laser diode module (emission wavelength of 640 nm, peak power 1W, Boston Electronics Inc.). The excitation wavelength has been changed from the previously used 660 nm to 640 nm in order to provide better spectral separation between fluorescence emission (ca. 680 nm) and elastically scattered excitation light (640 nm). The experiments revealed that the amount of background scattered light in the return signals has been reduced by a factor of ca. 5 that was a critical improvement for measurements in turbid, highly scattering waters.

The laser diode module is controlled by a custom electronic driver and generates, at a high repetition rate (from 50 to 200 Hz), a sequence of the FIRe flashes. The maximum achievable sampling rate is determined by the duration of a single-shot FIRe protocol and the time required to transfer and to store the data.

The laser beam is expanded and collimated to ensure the spot size of ca. 1cm on the coral surface. To ensure efficient collection of induced fluorescence, the aperture of the optical scheme has been increased to 10 cm (compared with 2 cm in the benchtop FIRe system). Although further increase in the aperture would improve the fluorescence signal, it is hard to be implemented without compromising the size of the sensor.

Fluorescence signals are separated by a band-pass interference filter (central wavelength of 690 nm and a half-band width of 20 nm) and recorded by a red sensitive avalanche photodiode module (Hamamatsu) with an incorporated amplifier and a temperature-dependent gain controller. The signal is further electronically filtered, digitized, and stored in onboard memory.

Figure 14. Laser-based Fluorescence Induction and Relaxation (FIRe) sensor for ROV/DSV platform. (Right) – Optical unit with a diode laser excitation source and large-aperture fluorescence collection optics; (Middle) – Electronic package with a computer board, control and data acquisition module, and power supply; (Left) – Housing for the electronic package.

5.4.2. Mechanical design – The mechanical assembly includes an optical module and an electronic package (Fig. 14). Each module is assembled into a standard polycarbonate Ikelite underwater housing rated for the depth up to 100 m. The two modules are connected with a waterproof cable which transfers a triggering signal to control the laser diode, an amplified fluorescence signal, and power between the two modules. Since different platforms might be used in future, the FIRe Sensor design and package is flexible enough to fit those platforms with minor modifications and adjustments. For example, the whole system can be readily assembled into a single underwater housing if needed.

5.4.3. Electronic package for the FIRe Sensor employs a PC/104-Plus ultra-low-power CPU board (CME137686LX333HR, Real Time Devices) coupled with a high-sampling-rate Analog/Digital Input/Output DataModule (DM7520HR, Real Time Devices). The latter is interfaced with a custom-designed electronic driver board that converts the digital signals from DM7520HR counters/timers to the high electric current pulses to drive the laser diodes. Also, the custom electronic board includes triggering circuitries for the laser driver and lock-in amplifiers, an electronic filter to eliminate the high-frequency noise and a gain-controlled amplifier for the recorded fluorescence signals. The gain is automatically controlled by the computer to adjust the recorded fluorescence signals to the optimal scale of the DM7520HR analog-to-digital converter.

The newly developed electronic package (Fig. 14) is twice as small in physical size than the previous electronic module of the diving FIRe instruments. Also, the use of faster, yet low-

power onboard computer allowed us to implement more sophisticated software codes for real-time data transfer and processing (see below).

5.4.4. Software procedure for data collection and storage has been improved to provide higher data flow on the fly. The rate of data collection has been further improved by using a PCI data bus for onboard data transfer.

With the goal of enhancing the capabilities for real-time automatic diagnostics of natural and anthropogenic stressors, we have upgraded the software package for FIRe fluorescence data analysis. The previous FIRe analysis procedure relies on the 10-parameters biophysical model and provides a comprehensive set of physiological characteristics. However, it is time consuming. It takes from 20 to 60 seconds for a single measurement to be processed, limiting its applicability for real-time analysis while diving. To overcome this limitation, we developed and implemented a short version of the analysis routine. It retrieves six (out of ten) most relevant parameters, but runs 20 times faster. The parameters include the minimal and maximum fluorescence yields (Fo and Fm), the quantum yield of photochemistry in Photosystem II (Fv/Fm), the cross-section of Photosystem II (σ_{PSII}), the energy transfer in photosynthetic antennae (p), and the first rate constant for the electron transport on acceptor side of Photosystem II (τ_{Qa}). This new routine allows the diver to obtain the preliminary results instantaneously and should help in monitoring and sampling in the field. Following the dive, all data files are downloaded and re-processed to retrieve the whole set of physiological parameters available from FIRe signals.

5.5. <u>Integration of FIRe fluorescence technology with Video Mosaics</u>

As part of this project, we collaborated with University of Miami (PI Dr. P. Reid, SERDP project SI-1333) to evaluate the potential for integrating FIRe technology with landscape mosaic technology developed by the Miami team. We have been asked by SERDP to consider how the respective approaches of the two projects might be integrated into a single strategy that can be used by DoD resource managers or their contractors to monitor coral reef health and to assess, when necessary, the impacts of particular stressors. SPAWARSYSCEN (San Diego, PI Bill Wild) assisted in the coordination of both projects to assess how they can better meet DoD needs.

During the collaborative effort with Dr. P. Reid (RSMAS, Miami Univ.), we examined and evaluated the following strategies to combine the technologies of the two projects. The advantages and potential limitations of each strategy have been examined. First, diver-operated FIRe instruments may be used to assess the photosynthetic and physiological characteristics of the dominant coral species, identified and selected based on mosaic mapping ("Separate Platform Approach"). The results of this monitoring may be potentially extrapolated to a larger scale using the data from mosaic surveys. Second, we examined how the FIRe Sensor for Diver Swum and ROV platforms could be integrated with the mosaicing system (a "Single Platform Approach").

On June 16-18, 2008, researchers from the University of Miami and Rutgers University conducted a joint sampling effort in the northern section of the Florida Reef Tract using both FIRe system and Video Mosaics. Two coral reefs sites were selected and surveyed jointly. At each reef, high-resolution video and still images were collected from a reef area roughly 10 x 10 m. The collected data have been used to construct a video mosaic of the sampled sites. In addition, 20-30 benthic organisms dominant in the area (including hard corals, soft corals, sponges, macroalgae) were identified within the area and FIRe fluorescence measurements were taken for each organism using the diver-operated FIRe instrument.

The first reef surveyed, Brooke's Reef, is located at 10 m depth and it has been surveyed previously by University of Miami researchers. Prior to the collection of the video mosaics, 30 benthic organisms were identified within the plot by deploying numbered tiles. The tiles were used to link the organisms within the video mosaic with the physiological measurements obtained for each one. After the completion of the image acquisition, researchers from Rutgers University collected fluorescence measurements for each of the organisms identified by the numbered tiles. The time required to collect the fluorescence information from 30 objects (with 3-4 replicates on each target) was approximately 40-60 min.

The same procedure was repeated at a second, shallower reef (5 m). On this site, 20 benthic organisms were identified with numbered tiles as described, and video images were collected. As described, fluorescence measurements of tagged benthic organisms were collected after the collection of the video. The two research groups worked well as a team and permanent sites were established and surveyed within two hours of bottom time. The FIRe measurements were evaluated and given a relative "health index" based on the 5 parameters obtained from individual FIRe measurements (Fo, Fm, Fv/Fm, sigma, and Tau-Qa). Health indices were separated into three groups; green, yellow, and red based on their FIRe values. Green indicated limited stress on the target organism, yellow indicated possible stress, and red indicated significant stress on the target organism. This field experiment resulted in a combined mosaic-FIRe product as shown in Figure 15.

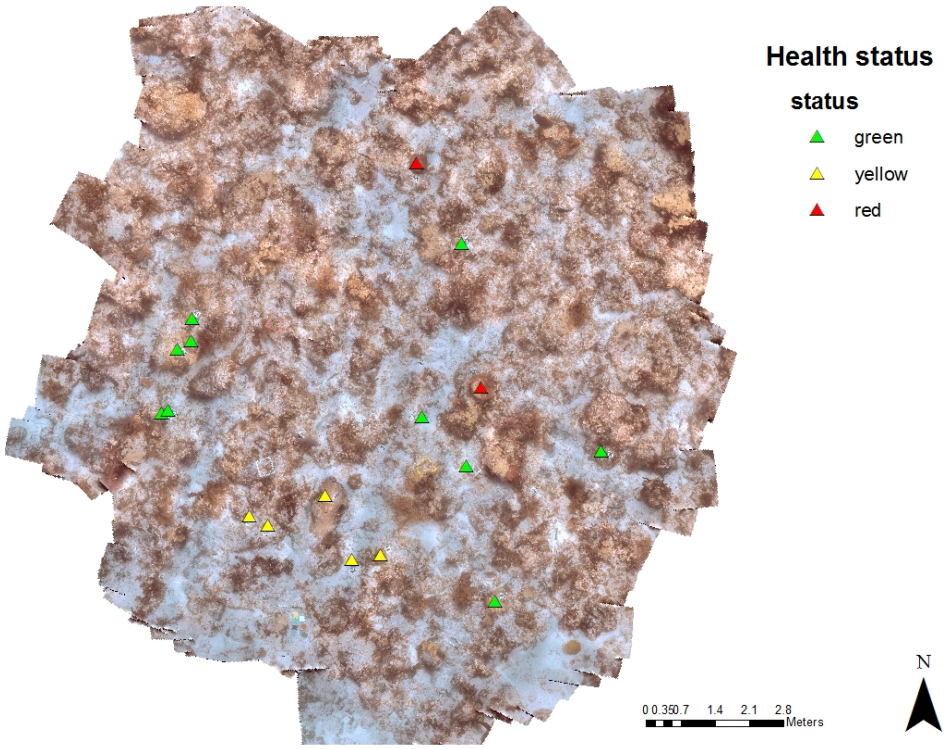

Figure 15. Combined mosaic/FIRe product. Locations of individual organisms examined with the underwater FIRe instrument are marked by triangles. The color of the triangles indicates the relative "health index" on the organism as estimated from FIRe measurements (green= healthy; yellow= potential stress; red= photosynthetic stress). The mosaics has been constructed by Dr. P.Read and A.Gleason (Univ. Miami).

6. Conclusions and Implications for Future Research

This research and development has provided advanced methods and ready-for-transition technology needed for the assessment of benthic ecosystems at DoD installations. We designed and developed a set of optical instruments, called Fluorescence Induction and Relaxation (FIRe) systems to assess the physiological status of benthic organisms including coral. 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 set of physiological parameters, it is capable of stress-specific diagnostics. For instance, the developed protocols were successfully employed to distinguish between common natural stresses (thermal stress, photoinhibition, or nutrient load) and selected anthropogenic stresses, such as metal and petroleum 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 (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 stressors in benthic ecosystems.

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 environmental applications, including the assessment of primary production and health of aquatic ecosystems, assessment of environmental stresses in marine and terrestrial plants, ecophysiology, photosynthesis research, forestry and agriculture, as well as water quality monitoring. In addition to this SERDP project, our custom FIRe systems have been employed during 15 oceanographic expeditions in the Atlantic, Pacific, and Southern Oceans to explore aquatic photosynthesis and primary production. The use of this technology provided unprecedented insight into the mechanisms and environmental factors that control primary production in the ocean.

Within the framework of the NOPP (National Oceanographic Partnership Program) funded project, we have developed a compact, ultra-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 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 has expressed its interest in commercializing this type of sensor.

A diver-operated FIRe system is proposed to be transferred to Naval Facilities Engineering Command Atlantic, via Naval Facilities Engineering Service Center for use at DoD coral reef sites (a proposed ESTCP project in partnership with Space and Naval Warfare Systems Center, San Diego).

We envision that our technology will be integrated into 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. Literature Cited

Falkowski PG, Koblizek M., Gorbunov M, and Kolber Z., (2004). Development and Application of Variable Chlorophyll Fluorescence Techniques in Marine Ecosystems. - In: "Chlorophyll a Fluorescence: A signature of Photosynthesis" (Eds. C.Papageorgiou and Govingjee), Springer, pp. 757-778.

Gorbunov MY, and Falkowski PG. 2004. Fluorescence Induction and Relaxation (FIRe) Technique and Instrumentation for Monitoring Photosynthetic Processes and Primary Production in Aquatic Ecosystems. In: "Photosynthesis: Fundamental Aspects to Global Perspectives" - Proc. 13th International Congress of Photosynthesis, Montreal, Aug.29 – Sept. 3, 2004. (Eds: A. van der Est and D. Bruce), Allen Press, V.2, pp. 1029-1031.

Gorbunov MY, and Falkowski PG. 2007. Analysis of Biophysical, Optical and Genetic Diversity of DoD Coral Reef Communities using Advanced Fluorescence and Molecular Biology Techniques (SERDP SI-1334) – Final Report to the Strategic Environmental Research and Development Program. June 2007.

Gorbunov M.Y., Falkowski P.G. and Kolber Z.S. 2000. Measurement of photosynthetic parameters of benthic organisms *in situ* using a SCUBA-based Fast Repetition Rate Fluorometer. - Limnol. and Oceanogr., 45: 242-245.

Gorbunov M.Y., Kolber Z.S., Lesser M.P. and Falkowski P.G. 2001. Photosynthesis and photoprotection in symbiotic corals. - Limnol. and Oceanogr., 46: 75-85.

Johnson-Worrell S. and M Gorbunov (2011). Effects of Sn on the Primary and Secondary Photosynthetic Reactions. - *Int. J. of Sustainable Water and Environmental Systems*, 2(No. 2): 97-102.

Kolber, Z., O. Prasil, and P.G. Falkowski 1998. Measurements of variable chlorophyll fluorescence using fast repetition rate techniques: defining methodology and experimental protocols. Biochem. Biophys. Acta 1367: 88-106.

Kuepper H., I Šetlík, M Spiller, FC Küpper, O Prášil (2002) – Heavy metal induced inhibition of photosynthesis: targets of in vivo heavy metal chlorophyll formation. - Journal of Phycology, V.38(3): 429–441.

Reid, R.P., S. Negahdaripour, D. Lirman, and P. Kramer. (2009) SI-1333 - Application of Landscape Mosaic Technology to Complement Coral Reef Resource Mapping and Monitoring. – Final Report to the Strategic Environmental Research and Development Program. December 2009.

Tchernov D, Gorbunov MY, de Vargas C, Yadav SN, Milligan AJ, Haggblom M, Falkowski PG. (2004) Membrane lipids of symbiotic algae are diagnostic of sensitivity to thermal bleaching in corals. – *Proc. Nat. Acad. Sci., U.S.A.*, 101 (37): 13531-13535.

Veron, J. E. N. (2000). Corals of the world (Townsville MC, Australian Institute of Marine Science).

SPAWARSYSCEN PAC. 2008. Proceedings from the Coral Reef Monitoring and Assessment Workshop, 18-19 November 2008, Miami, Florida. Prepared for the Strategic Environmental Research and Development Program and Rosenstiel School of Marine and Atmospheric Sciences by SPAWARSYSCEN PAC, San Diego, California.

Appendix A

Publications

Journal Articles

1. Helman, Y., F. Natale, R.M. Sherrell, M. LaVigne, V. Starovoytov, M.Y. Gorbunov, P.G. Falkowski. (2008) Extracellular matrix production and calcium carbonate precipitation by coral cells in vitro. - *Proc. Natl. Acad. Sci USA*, 105: 54-58.

2. Vittadello M, MY Gorbunov, DT Mastrogiovanni, LS Wielunski, EL Garfunkel, F Guerrero, D Kirilovsky, M Sugiura, AW Rutherford, A Safari, and PG Falkowski (2010). Photoelectron Generation by Photosystem II Core Complexes Tethered to Gold Surfaces. – *ChemSusChem*, Volume 3 Issue 4, Pp. 471 - 475.

3. Johnson-Worrell S. and M Gorbunov (2011). Effects of Sn on the Primary and Secondary Photosynthetic Reactions. - *Int. J. of Sustainable Water and Environmental Systems*, 2(No. 2): 97-102.

4. Yan C, O Schofield, Z Dubinsky, D Mauzerall, P Falkowski, and M Gorbunov (2011) Photosynthetic energy storage efficiency in Chlamydomonas reinhardtii, based on microsecond photoacoustics – *Photosynthesis Research*, in press.

5. Tchernov D., H Kvitt, L Haramaty, TS Bibby, MY Gorbunov, H Rosenfeld, and PG Falkowski (2011). Apoptosis and the selective survival of host animals following thermal bleaching in zooxanthellate corals - *Proc. Natl. Acad. Sci USA,* June 14, 2011, vol. 108(24), pp. 9905-9909.

6. Gorbunov M.Y., Kuzminov F.I., Fadeev V.V., Dongun Kim J., Falkowski P.G. (2011). A Kinetic Model of Non-Photochemical Quenching in Cyanobacteria – *Biochem Biophys Acta: Bioenergetics*, in press.

Conference Presentations

7. Johnson S., Gorbunov M., and Falkowski P.(2008) - The effect of heavy metal contamination on the photosynthetic characteristics of diatoms and zooxanthelae. – 2008 Gordon Conference on Photosynthesis – June 22-27, 2008.

8. Gorbunov M., D. Tchernov, F. Natale, S. Johnson, P. Falkowski (2008) - Development and application of variable fluorescence techniques and instrumentation for monitoring and assessing coral reefs - 11th International Coral Reef Symposium, July 7-11, 2008, Ft.Lauderdale, FL, p.16-5.

9. Howard R., P.Falkowski (2008) Quantum Efficiency for Light Enhanced ROS Production in Corals - A Physiological Approach to Predict Bleaching. - 11th International Coral Reef Symposium, July 7-11, 2008, Ft.Lauderdale, FL, p.5-15.

10. Gorbunov MY and PG Falkowski (2008) – The application of lifetime analyses to the interpretation of satellite-based, solar induced chlorophyll fluorescence signals. – NASA Carbon Cycle Conference, April 28-May 02, 2008, College Park, MD.

11. Gorbunov, M. and Falkowski, P. (2008) Development and applications of Florescence Induction and Relaxation technology for assessment and monitoring of coral reef communities. - SERDP Coral Reef Monitoring and Assessment Workshop, Miami, FL, November 18-19, 2008.

12. Lirman, D., Reid, R.P., Gorbunov, M., Falkowski, P. (2008) Landscape Video Mosaic and Florescence Induction and Relaxation System Integration. SERDP Coral Reef Monitoring and Assessment Workshop, Miami, FL, November 18-19, 2008.

13. Gorbunov MY, L.Haramati, F.Natale, S. Johnson, and PG Falkowski (2008) – Development and Applications of advanced fluorescence technique for assessment of physiological status and health of corals at DoD installations – SERDP/ESTCP Conference "Partners in Environmental Technologies", Dec 2-4, 2008, Washington, DC.

14. Gorbunov MY, S. Johnson, D. Tchernov, and PG Falkowski (2009) - Development of Bio-Optical Techniques for Monitoring and Assessing DoD Coral Reefs - Partners in Environmental Technologies Technical Symposium and Workshop, Dec 1-3, 2009, Washington, DC.

15. Kuzminov F., E. Maximov, M. Gorbunov, and V. Fadeev. (2010) - Fluorescent diagnostics of cyanobacteria. - «SPIE Photonics Europe», 2010, April12-16, Brussels, Belgium.

16. Gostev T., F. Kuzminov, M. Gorbunov, and V. Fadeev (2010) - Phytoplankton as a fluorescent bioindicator of ecotoxicants in natural waters. - «SPIE Photonics Europe», 2010, April12-16, Brussels, Belgium.

17. Kuzminov F., E. Maximov, M. Gorbunov, V. Fadeev. (2010) - Diagnostics of cyanobacteria and zooxanthellae using nonlinear laser fluorimetry and variable fluorescence techniques. - ASLO Joint Meeting "Aquatic sciences: global changes from the center to the edge", June 6–11, 2010, Santa Fe, NM, p 52.

18. Gorbunov MY; Falkowski PG (2010) – Development and applications of picoseconds kinetic fluorometry to study fluorescence lifetimes and photosynthetic processes in oceanic ecosystems. - ASLO Joint Meeting "Aquatic sciences: global changes from the center to the edge", June 6–11, 2010, Santa Fe, NM, (Abstract ID:7518).

19. Kuzminov F., E. Maximov, M. Gorbunov, V. Fadeev. (2010) - Studying of photoprotection mechanisms in photosynthetic apparatus of cyanobacteria using nonlinear laser fluorometry and variable fluorescence techniques. - XII International Conference on Laser Applications in Life Sciences, LALS–2010, June 9–11, 2010, Oulu, Finland. Book of Abstracts, p. 267.

20. Gostev T., F. Kuzminov, M. Gorbunov, V. Fadeev. (2010) - Application of nonlinear laser fluorimetry for study of photophysical processes in photosynthetic organisms. - XII International Conference on Laser Applications in Life Sciences, LALS–2010, June 9–11, 2010, Oulu, Finland. Book of Abstracts, p. 273.

21. Cervino JM, M Gorbunov, K Hughen, KB Strychar, P Lawther, B Hauff, J Kneeland, K Furby, A Dona, A Wier, R.L. Hayes, and T.J. Goreau. (2010) - The thermal and pathogenic effects of Vibrio species on tropical reef corals and their Symbiodinium clade phylo-types in situ and in vitro. - Vibrios In the Environment 2010, 7-12 November 2010, Biloxi, Mississippi.

22. Johnson S., Gorbunov MY, Kuzminov F., Fadeev V., Haramati L., and P. Falkowski (2010) - Development of Bio-Optical Techniques for Assessing Heavy Metal Toxicity in Aquatic Ecosystems – "Partners in Environmental Technology Technical Symposium & Workshop", Washington, D.C., Nov.30-Dec.2, 2010, P461

23. Johnson-Worrell S. and M Gorbunov (2010) - Effects of Sn on the Primary and Secondary Photosynthetic Reactions. - International Conference on Energy, Water & Environment (ICEWE 2010) Amman, Jordan, December 12-15, 2010.

24. Gorbunov MY, Johnson S., and P. Falkowski (2010) - Novel Fluorescence Technology for Assessing the Impact of Petrochemicals on Aquatic Ecosystems and for Rapid Bioassay of Petroleum Toxicity – "Partners in Environmental Technology Technical Symposium & Workshop", Washington, D.C., Nov.30-Dec.2, 2010, P462

25. Olson B., J Harrold, MY. Gorbunov, DT. Mastrogiovanni, LS. Wielunski, E Garfunkel, W. Rutherford, and Michele Vittadello (2011) - Indirect Electrochemistry of Photosystem II Core Complexes Tethered to Gold Surfaces. - 28th Eastern Regional Photosynthesis Conference, April 1-3, 2011, Marine Biological Laboratory, Woods Hole, MA

26. Gostev T., V. Fadeev, M. Gorbunov (2011) - Study of the Non–Photochemical Quenching of Chlorophyll-a Excited States in Algae using Variable Fluorescence and Nonlinear Laser Fluorimetry. - International Workshop "Mechanisms of Non-Photochemical Quenching", 6–10 April 2011, Passau, Germany.

27. Gorbunov M., F. Kuzminov, V. Fadeev, P. Falkowski (2011) - A Multistep Kinetic Model of Non–Photochemical Quenching in Cyanobacteria - International Workshop "Mechanisms of Non–Photochemical Quenching", 6–10 April 2011, Passau, Germany.