*Ref. No. [UMCES] CBL 2019-007*
*ACT VS19-02*

# PERFORMANCE VERIFICATION STATEMENT
# For Turner Designs CyanoFluor

**TECHNOLOGY TYPE:** Multi-spectral Fluorometers

**APPLICATION:** In situ estimates of algae for coastal moored deployments

**PARAMETERS EVALUATED:** Accuracy, precision, range response and reliability

**TYPE OF EVALUATION:** Laboratory and Field Performance Verification

**DATE OF EVALUATION:** Testing conducted from June 2017 to November 2017

**EVALUATION PERSONNEL:** T.H. Johengen, H. Purcell, G.J. Smith, D. Schar, H. Bowers, M. Tamburri, D. Fyffe and G.W. Jeter.

.

**NOTICE:**

ACT verifications are based on an evaluation of technology performance under specific, agreed-upon protocols, criteria, and quality assurance procedures. ACT and its Partner Institutions do not certify that a technology will always operate as verified and make no expressed or implied guarantee as to the performance of the technology or that a technology will always, or under circumstances other than those used in testing, operate at the levels verified. ACT does not seek to determine regulatory compliance; does not rank technologies nor compare their performance; does not label or list technologies as acceptable or unacceptable; and does not seek to determine "best available technology" in any form. The end user is solely responsible for complying with any and all applicable federal, state, and local requirements.

This document has been peer reviewed by ACT Partner Institutions and a technology-specific advisory committee and was recommended for public release. Mention of trade names or commercial products does not constitute endorsement or recommendation by ACT for use.

Questions and comments should be directed to:

Dr. Tom Johengen
ACT Chief Scientist
CIGLR - University of Michigan
4840 S. State Street
Ann Arbor, MI 48108 USA
Email: Johengen@umich.edu

## TABLE OF CONTENTS

## EXECUTIVE SUMMARY

The Alliance for Coastal Technology (ACT) conducted a sensor verification study of in situ multispectral fluorometers during 2017-2018 to characterize performance measures of accuracy and reliability in a series of controlled laboratory studies and field tests in diverse coastal environments. Laboratory tests using known algal cultures both individually and in various combinations along with add-in matrix challenges for turbidity and CDOM were conducted at Moss Landing Marine Laboratory (MLML) and NOAA Great Lakes Environmental Research Laboratory (GLERL). In total, 40 different exposure trials were conducted within these Lab tests. Five different field testing applications were conducted including three continuous underway surface mapping cruises and two moored deployments. Underway mapping cruises were conducted in San Francisco Bay, in Monterey Bay, and in western Lake Erie. Underway cruises covered between 75 – 80 km and each included seven isolated tank-exposure comparisons comprised of two timepoints over 30 minutes. The first moored field test was conducted over 13 days in a flow-through tank using Maumee River source water at the Bowling Green Municipal Water Treatment Plant. The second mooring test was conducted for 28 days from a submerged rack deployed off the research pier of the Chesapeake Biological Research Lab in Solomons, MD. Instrument performance was evaluated against reference samples collected and analyzed by ACT staff or through sub-contracts at certified Phytoplankton counting laboratories at the University of Minnesota Duluth's Natural Resources Research Institute and the Smithsonian Environmental Research Center. Instrument performance was evaluated against extracted chlorophyll, extracted phycocyanin, and algal species classification at the functional group level on the basis of estimated biovolume contribution within each sample. A total of 243 reference samples were collected for direct instrument comparisons. For each reference sample six replicates were filtered for pigment analysis with two replicates analyzed for chlorophyll and three replicates analyzed for phycobilins. One filter was reserved in storage and used when the variance in analytical replicates exceeded a 10 percent threshold. Field duplicates and field trip blanks were collected during each test application as a measure of Quality Assurance.

This document presents the results of the Turner Designs CyanoFluor Handheld HAB Indicator configured for *in vivo* fluorescence detection from chlorophyll (CHL) and phycocyanin (PC) pigments in natural water samples. CyanoFluor calculates and reports a PC to CHL ratio that indicates what portion of the total phytoplankton population is comprised of PC-containing algae (cyanobacteria), algae that are known to cause Harmful Algal Blooms (HABs). The CyanoFluor is factory calibrated; therefore, no calibration standards or tools are required or necessary to calibrate this instrument. The CyanoFluor also provides raw fluorescence readings that can be correlated with, and reported as µg/L chlorophyll concentrations with proper empirical calibration. For this evaluation, all comparisons were done using raw fluorescence values (RFU).

The fluorescence response of the CyanoFluor is summarized below for each of the Lab and Field tests conducted within the evaluation based on a linear regression of instrument values versus extracted reference sample pigment concentrations. Response slopes for both chlorophyll (CHL) and phycocyanin (PC) varied across individual algal culture species in the lab tests and across the different field deployments. Response slopes for CHL were lower in lab tests where CDOM addition occurred since no attempt was made to blank correct for CDOM. For field tests, the lowest CHL response slope (17.9) was seen in western Lake Erie during a significant colonial *Microcystis* bloom and the highest response slope (466) was seen in the Chesapeake Bay deployment where

species are small and the composition more diverse. Phycocyanin response slopes were much more consistent over the range of diverse field tests; however, significant variation was seen in the Lab tests. The variability in PC response, and subsequently in the PC:CHL ratio, is somewhat difficult to interpret because often PC concentrations were quite low (less than 2 µg/L).

| Test | CHL Response Slope | CHL Regression R-squared | PC Response Slope | PC Regression R-squared | # Ref Samples | Reference PC:CHL Max |
|---|---|---|---|---|---|---|
| ***LAB Tests*** | | | | | | |
| ML Test1 | 364 | 98 | 177 | 71 | 8 | 0.08 |
| ML Test 2 | 452 | 99 | nr | nr | 10 | 0 |
| ML Test 3 | 156 | 96 | 308 | 92 | 31 | 0.05 |
| ML Test 4 | 417 | 99 | 84.8 | 95 | 19 | 0.24 |
| ML Test 5 | 313 | 98 | 108 | 95 | 21 | 0.25 |
| GLERL | 134 | 66 | 32.3 | 82 | 73 | 1.4 |
| ***Field Test Underway*** | | | | | | |
| SF Bay | 116 | 96 | 267 | 1.3 | 99 | 0.03 |
| Monterey Bay | 82 | 30 | 114 | 11 | 99 | 0.03 |
| WLE | 17.9 | 42 | 112 | 84 | 100 | 0.85 |
| ***Field Test Moored*** | | | | | | |
| Maumee R | 121 | 92 | 97.3 | 86 | 36 | 0.18 |
| Chesapeake Bay | 466 | 78 | 66.7 | 55 | 19 | 0.35 |

**nr: denotes no result computed*

***The manufacturer was given the opportunity to respond to the findings and presentation of this evaluation and their response is provided at the end of the report.***

## BACKGROUND AND OBJECTIVES

ACT was established on the premise that instrument validation of existing and emerging technologies is essential to support both coastal science and resource management. The overall goal of ACT's verification program is to provide industry with an opportunity to have a third-party test their instruments in both controlled laboratory settings and in diverse field applications across a range of coastal environments in order to provide users of this technology with an independent and credible assessment of instrument performance. To this end, the data and information on

performance characteristics were focused on the types of information users most need. It is important to note that ACT does not certify technologies or guarantee that a technology will always, or under circumstances other than those used in testing, operate at the levels verified. ACT does not seek to determine regulatory compliance; does not rank technologies or compare their performance; does not label or list technologies as acceptable or unacceptable; and does not seek to determine "best available technology" in any form.

As part of our service to the coastal community, ACT conducted a performance verification of commercially available, in situ multi-excitation fluorometers that are designed to discriminate among classes of phytoplankton and may be used to enhance the detection of harmful algae and cyanobacteria. The fundamental objectives of this Performance Verification were to: (1) highlight the potential capabilities of particular in situ fluorometers for monitoring harmful algal blooms; (2) verify the claims of manufacturers on the performance characteristics of these instruments when tested in a controlled laboratory setting, and (3) verify performance characteristics of these instruments when applied in real world applications in a diverse range of coastal environments.

## INSTRUMENT TECHNOLOGY TESTED

CyanoFluor™ Handheld HAB Indicator is configured for *in vivo* fluorescence detection from chlorophyll (CHL) and phycocyanin (PC) pigments in natural water samples. CyanoFluor calculates and reports a PC to CHL ratio that indicates what portion of the total phytoplankton population is comprised of PC-containing algae (cyanobacteria), algae that are known to cause Harmful Algal Blooms (HABs). PC fluorescence changes proportionally with the abundance of cyanobacteria; therefore the fluorescence from PC can be used to estimate the relative abundance of cyanobacteria in water samples. For example, when cyanobacteria are not present in natural water samples, the fluorescence from PC is very low, yielding ratios less than 0.1; when cyanobacteria make up 50% of the total phytoplankton population the PC:CHL ratio can be 0.5 or greater. Monitoring PC to CHL ratios over time will indicate whether environmental conditions are changing, favoring cyanobacterial production, potentially leading to cyanobacterial blooms. This monitoring effort can be used to predict the onset of HABs and alert users so the proper protocols can be implemented to prevent or reduce harmful bloom effects. The CyanoFluor also provides raw fluorescence readings that can be correlated with, and reported as µg/L chlorophyll concentrations with proper empirical calibration. The CyanoFluor is factory calibrated; therefore, no calibration standards or tools are required or necessary to calibrate this instrument. Solid state optoelectronics ensure the instrument will remain stable over time. Water samples can be quickly analyzed with results displayed in less than 30 seconds. Simply insert a 10x10mm square glass or quartz cuvette with your sample and press READ. All displayed results are automatically logged and can be downloaded at a later time. The CyanoFluor benefits include: simple intuitive functions; self-contained design; small size; total data storage capacity of 1,000 measurements; and very low power draw (4-AAA batteries).

## PERFORMANCE EVALUATION TEST PLAN

Phytoplankton and cyanobacteria offer a range of inherent characteristics that enable their discrimination and classification. Their morphological and cell surface diversity enables broad discrimination through microscopic examination and light scattering properties. Photosynthetic pigment composition is also taxon specific and their inherent absorption and fluorescence properties provide an additional, sensitive target for *in situ* detection and discrimination. This verification study evaluated the field and laboratory performance of instruments leveraging the capacity for fluorescence-based parsing of phytoplankton community composition. Evaluations focused on the ability of these technologies to determine presence and abundance of cyanobacteria and potentially harmful eukaryotic phytoplankton (diatoms, dinoflagellates, prymnesiophytes) within mixed natural communities.

A single instrument was provided to ACT by Turner Designs and used in all subsequent testing without any further calibration or servicing by the company. Prior to testing, all ACT personnel participated in a full day training session from the manufacturer in set-up and operations. Since testing was performed in many different environments and algal communities, no effort was made to optimize performance or calibration for any given test.

### Laboratory Tests

Laboratory tests of response linearity, precision, range, and reliability were conducted at Moss Landing Marine Laboratories (MLML). First, instrument output was referenced to defined Basic Blue 3 (BB3) and/or Rhodamine WT (RWT) concentrations under standard reference conditions by immersion in one or two-point standardized solutions. Second, instrument response to several individual freshwater and marine cultures was quantified at several concentration levels. Third, instruments were exposed to mixtures of different phytoplankton assemblages within freshwater and marine cultures. Lastly, matrix effects of turbidity and dissolved organic carbon were assessed through addition to the range of mixed assemblage concentrations. *It should be noted that many of the lab cultures were contaminated and we were not able to quantify specific response functions to individual algal taxon.*

The various test conditions were produced in well mixed (via mechanical mixing), temperature controlled water baths where instruments were submerged for testing. Test tanks were equipped with a multi-parameter YSI EXO2 sonde to continuously monitor temperature, salinity, turbidity, fDOM, pH, DO, CHL, and BGA during all laboratory testing. All laboratory tests were conducted at a fixed temperature and salinity level near the closest optimal growth temperature for all phytoplankton taxa utilized. Fluorometric response and discrimination were tested on both freshwater and marine algal species utilizing cultures of live algae added into a background matrix of filtered deionized water or seawater, supplemented with appropriate salt and nutrient additives ( BG11+Si and L1 respectively) at known mixtures and concentrations. Freshwater and seawater were obtained from the MLML aquaculture facility.

*Phytoplankton Taxa* – Sources of the algal cultures came from a variety of sources including the traceable commercial sources UTEX and NCMA, however, when those stocks did not propagate well in large batch cultures, additional cultures from personal collections at the NOAA Great Lakes Environmental Research Lab were included. Freshwater taxa from NOAA collections included a cyanobacteria (*Microcystis* spp.), a chlorophyte (*Coelastrum*), and a dinoflagellate

(*Peridinium*). Marine taxa generated from the NCMA collection included a diatom (*Thalassiosira* spp.), a dinoflagellate (*Amphidinium carterae*), and a cyanobacteria (*Synechococcus* spp.). Cultures were grown in large 20L batch cultures under cool LED light (ca 75 µmol quanta $m^{-2}$ $s^{-1}$) using 16-8 light dark cycles at 20$^{o}$C) using appropriate growth media as indicated above to mid-log phase (determined by cell counts).

*Response Linearity and Range* – For linearity or range tests, comparative measurements of instrument and reference samples were generated from instrument readings at 10 second intervals, after the instruments were allowed at least 15 minutes to equilibrate to each new test condition changes. The instrument mean and standard deviation (SD) were computed from readings averaged around a one-minute interval for each reference sample timepoint. For each test condition two or three reference samples were taken at roughly five-minute intervals following the equilibration period. Each reference sample was analyzed for CHL, PC, PE, and algal biovolume as described below. Mixtures of phytoplankton taxa were titrated based on stock cultures' volumetric chlorophyll concentration. Given that taxa vary according to their pigment quotas, it is recognized that actual cell densities will not be present in the ratios defined, and that the ratios are based on pigment content. All additions and test conditions were maintained at low ambient light (< 75 µmol photon $m^{-2}$ $s^{-1}$). Individual species were added sequentially to produce different ratios and concentrations of algae. The exact CHL concentrations tested varied dependent on culture yields however, 'real-world' ranges were targeted. A regression of instrument fluorescence versus total CHL was examined to estimate the potential linear environmental detection range.

*CDOM and Turbidity Challenges* – Sensitivity to water clarity and natural fluorescence was assessed by exposing the test instruments to varying levels of background CDOM (Pahokee Peat leachate reference material) and turbidity (Elliot Silt Loam reference material). Instruments were initially placed in a test bath at 20 $^{o}$C and fluorescence response measured at three algal concentrations over 15-minute exposures, after which, several additions of CDOM and turbidity were sequentially produced to examine effects on response. For some tests, following the CDOM and turbidity additions, additional algal culture was added to examine instrument linearity under the matrix challenge conditions. For each challenge condition, the tank was equilibrated for 15 minutes to insure uniform mixing (T0), followed by 15 minutes of instrument measurements (T15) for analysis against reference samples. Continuous monitoring of CDOM and turbidity within the test tank was conducted at one-minute intervals with the EXO sonde to verify the stability of the test conditions. Challenge CDOM concentrations were increased from background to levels ranging from 2 - 20 mg/L (as DOC) and turbidity increased to levels between 10 - 100 NTU. Turbidity concentrations of the discrete reference samples were measured using a Hach 2100 benchtop turbidity sensor calibrated in NTU. CDOM concentrations on the discrete reference samples were measured on filtered reference samples analyzed by absorbance spectroscopy (see below).

*Reliability* – Instrument reliability during the laboratory test was determined by comparing percent of data recovered versus percent of data expected. Comments on problems or instrument failures were noted.

Due to contamination of several of the freshwater and marine cultures, we were not able to conduct the intended single species responses and not all additions followed the exact described method due to time and handling constraints. The actual conditions of each trial within a daily lab test are presented at the beginning of the results for each lab test for clarity. In addition, a second lab test was established at the NOAA Great Lakes Environmental Research Lab using clean,

mono-culture freshwater algal cultures. For this test instruments were exposed to four individual species in small 2L batches, followed by mixtures of all species together at four different composition ratios, and finally a repeat of the last mixture with CDOM and turbidity enhancements at similar levels the previous lab test.

## Field Testing

A rigorous field testing component was conducted to provide a variety of algal composition and densities within various ecosystems including riverine, lake, estuarine, and marine. Exact environmental conditions were constrained by the available testing windows available at each site, but the schedule was designed to maximize the potential of including exposure to known harmful algal bloom communities within each field deployment. Instrument performance and reliability was determined in both moored and surface mapping applications. Instrument reliability for each of the field tests was assessed by comparing percent of data recovered versus percent of data expected. Comments on problems or instrument failures were recorded.

### *Moored Deployment*

*In situ* evaluations of instrument performance in a moored application were conducted at two ACT Partner Institution sites. The first moored deployment was conducted in a flow-through tank sampling water from the Maumee River at a location adjacent to the City of Bowling Green, OH, public water utility. The deployment occurred over 13 consecutive days and provided a wide range of chlorophyll concentrations (10 to 120 µg/L), high turbidity (up to 100 NTU), and variable concentrations of cyanobacteria. A second moored application was conducted at the Chesapeake Biological Laboratory in Solomons, MD. Instruments were deployed on a dockside mooring in Chesapeake Bay for 28 continuous days. Test conditions provided a range of salinity and temperature conditions and variable algal composition and abundance as a function of tidal cycle and variable riverine input. This environment is also known for high rates of both soft and hard biofouling, and an additional objective of this test application was to evaluate the ability of the *in situ* instruments to maintain performance levels under high biofouling.

Field Testing Procedures

*Instrument Setup* - Prior to deployment, all instruments were setup and calibrated at the field sites as suggested by the manufacturer. Fluorometers were programmed to record data at a minimum frequency of every 15 minutes during the entire field deployment. All internal clocks were set to local time using www.time.gov as the time standard. Before deployment, all instruments were exposed to a DI blank and two concentrations of BB3 (0.05 and 0.5 µg/mL) dye produced from a common stock prepared and distributed by MLML. Responses to the dye exposure were used to ensure good working order and establish any calibration offset across different test applications. Photographs of instruments were taken just prior to deployment and just after recovery to provide a qualitative estimate of biofouling during the field tests.

*Deployment Rack* - All test instrument packages were deployed side-by-side on a common mooring rack such that all sensor measurement windows were at the same depth. Instrument sensor heads were deployed with a separation distance of at least one instrument-diameter to minimize the potential for cross interference. For the Maumee River test, instruments were deployed in a 500 L, 1 m deep flow-through tank with sensor heads at approximately 20 cm off the bottom. For the CBL moored deployment, the rack was deployed so that all of the fluorometers remained a minimum of 1 m below the water surface, accounting for variance due to tidal state or

river stage. For each deployment a calibrated CTD and/or a multi-parameter EXO2 sonde was attached to the mooring and programmed to provide an independent record of temperature, conductivity, CDOM, turbidity, CHL, and PC at the same depth and the same 15-minute intervals as the test instruments. For the CBL deployment, light intensity was also measured continuously with a LI-COR LI-193 underwater spherical PAR sensor mounted on a Seabird SBE911 CTD at the same depth as the sensors.

*Sampling Schedule* – For the Maumee River deployment we collected two references samples per day approximately one hour apart during the workweek, however, once each week we sampled four times within a day to capture a larger daily range. When possible we varied the sampling timepoints between morning and afternoon on different days to capture some variation in light history. For the CBL deployment, we evaluated diurnal responses across the day-night spectrum on three occasions including day 2, day 3, and day 27 of the deployment. On those days, we collected four reference samples throughout the day at instrument sampling timepoints: 06:00, 10:00, 15:00, and 20:00. During all other sampling events, reference samples were collected twice a day with one in the morning and one in the afternoon.

*Water Samples* – At the Maumee River test site reference samples were collected by dipping two 1 L polypropylene bottles directly into the tank. Bottles were rinsed a minimum of three times before final collection. At CBL reference samples were collected with a standard 4-liter Van Dorn bottle. The sampling bottle was lowered into the center of the sensor rack at the same depth and as close as safely possible to the fluorometers and allowed to soak for one minute prior to sample collection. The bottle was triggered to close at the same time as instrument sampling to ensure that the same water mass was being evaluated. For each reference sample, six replicates (two for CHL, three for PC/PE, one reserve) were filtered under low light and low vacuum conditions, and stored in a -80 $^{o}$C freezer until analysis (methods described below). Cell abundances of coarse taxonomic groupings (e.g. diatoms, dinoflagellates, chlorophytes, cyanophytes, others) and biovolumes were determined on Lugol fixed sample aliquots. A whole water subsample was collected to measure turbidity using a Hach model 2100AN Turbidometer. Lastly, filtrate was collected using acid-cleaned filters and shipped to MLML for CDOM analysis. Field duplicates were collected during one sampling event per week at each test site. Duplicates were collected by deploying two Van Dorn bottles (or two dipped 1 L bottles) side-by-side, and were processed in identical fashion.

### *Surface Mapping Deployment*

*In situ* evaluations of instrument performance in surface mapping applications were conducted at three locations including freshwater, estuarine, and marine environments. On July 6$^{th}$ a surface mapping cruise was conducted in San Francisco Bay in collaboration with Dr. Raphe Kudela of UC Santa Cruz and Dr. Jim Cloern of USGS following their existing HAB survey program sampling over a 150km transect ranging from Palo Alto in the south to the Richmond bridge in the north. On July 13$^{th}$ a second surface mapping cruise was conducted in Monterey Bay over a transit distance of 75km covering a range from outside the harbor to open ocean environments. On August 13$^{th}$ the third surface mapping cruise was conducted in the western basin of Lake Erie during a known period of *Microcystis* blooms. The survey covered approximately 75 km of transit and included regions dominated by cyanobacteria near the mouth of the Maumee River to regions further offshore to the north and east with lower abundance and a more diverse composition.

*Instrument Setup* - Prior to deployment, all instruments were exposed to DI and the two BB3 dye standards. Test instruments were programmed to record data at 1-second intervals. Submersible instruments were deployed in a flow-through tank with a known exchange rate (nominally 10-15 min). The tank was kept shaded under cover. A calibrated multi-parameter sonde was positioned within the tank to provide an independent record of temperature, conductivity, CDOM, turbidity, CHL, and PC continuously at 1-minute measurement intervals.

*Water Samples* – Seven or eight stations were selected during each surface mapping survey to make comparative reference sample measurements. Stations were selected to cover a diversity of phytoplankton abundance and composition. At each selected station, water in the flow-through tank was isolated for a period of 30 minutes, keeping it well mixed with manual stirring. After an initial equilibration period of 15 minutes, reference samples were taken at timepoints of 20 and 30 minutes from the point of isolation. Sub-samples of the composited sample draw were used to expose the one bench-top test instrument. Samples were collected under shade to minimize light exposure and immediately taken into a shipboard laboratory (or a shaded deck space for Lake Erie) and processed using the same protocols as defined for the field mooring deployments. Reference samples were analyzed for extracted chlorophyll a and phycobilins, fixed cell counts, CDOM, and turbidity as described below.

## Reference Sample Analysis

### *Pigment Quantification*

Water samples were collected onto 25 mm Whatman GF/F filters under low vacuum filtration (<5 in Hg). Filtered volumes (sufficient to discern coloration of filters) varied by sampling location (≥ 100 mL). Chlorophyll a (CHL) content of the filtered material was determined by fluorescence analysis of dimethylformamide (DMF) extracts using the non-acidification method (Speziale et. al. 1984) on a Turner Designs 10 AU fluorometer calibrated against certified chlorophyll a standard (Turner Designs). Phycobilin (phycocyanin, PC and phycoerythrin, PE) content of filtered water samples was determined by fluorescence analysis of phosphate buffer (50 mM, pH 6.8) extracts following 3 freeze-thaw cycles and sonication to maximize pigment extraction (Lawrenz et al. 2011) on a Turner Aquaflor fluorometer calibrated with certified PC and PE standards (Prozyme Inc.). All sample handling for pigment extraction was conducted under low light to minimize sample degradation. All fluorometer calibrations and extract readings were done at room temperature that was typically controlled at 20 ± 1.0 $^{o}$C.

A total of six replicates was filtered for each reference sample and stored at -80 $^{o}$C immediately after processing. Filters for chlorophyll were stored and extracted in amber glass vials. Filters for PC/PE were stored and analyzed in 15 ml opaque, polycarbonate centrifuge tubes. Pigment analysis was conducted on two replicates for chlorophyll and three replicates for phycobilins. One filter was reserved in storage at -80 $^{o}$C and subsequently analyzed when the variability in the initial results were above a threshold of 20% in coefficient of variation. All reference sample pigment analysis was performed by the same trained ACT personnel using the same instrumentation and procedures.

### *Species Identification, Abundance and Biovolume*

Whole water samples (500 mL) were fixed with acidified Lugol's (1% final concentration, v/v) and concentrated as necessary by settling or gentle centrifugation (3000 rpm, 10 min). Total

cell abundance was enumerated microscopically and assigned to coarse taxonomic groups (i.e. diatoms, dinoflagellates, chlorophytes, prymnesiophytes, and cyanobacteria), or to the lowest taxonomic category needed to assign appropriate biovolume conversions. Cell abundance was converted to biovolumes using site-specific dimensional relationships based on equivalent spherical diameter. Data are reported as total phytoplankton abundance and biovolume of each group after adjustment for volume dilutions.

For the surface mapping survey in San Francisco Bay phytoplankton abundance was determined from image libraries generated with an Imaging FlowCytobot (IFCB) operated by UC Santa Cruz personnel. For field sampling in Monterey Bay and San Francisco Bay, additional sub-samples were preserved with paraformaldehyde at a final concentration of 0.24% and evaluated using flow cytometry. For these test sites all phytoplankton analysis and cytometric quantification was performed by ACT staff at the MLML based on local knowledge and experience in these analyses.

For the Great Lakes tests, phytoplankton counting was conducted under a contract to Dr. Euan Reavie of the National Resources Research Institute in Duluth, MN. The SOPs for counting Great Lakes samples follow protocols of the USEPA Great Lakes National Program Office (GLNPO) Biological Surveillance Program which has been in place for over thirty years. Details of the SOPs may be found at: http://www3.epa.gov/greatlakes/monitoring/sop/chapter4/lg401.pdf. For the Chesapeake Bay tests, phytoplankton counting was conducted under a contract to Tim Mullady of the Smithsonian Environmental Research Center in Edgewater, MD. Phytoplankton analyses were conducted using an Utermohl settling chamber and inverted phase/fluorescent microscope following the Maritime Environmental Resource Center SOP entitled, Live Organisms ≥ 10 to < 50 µm Standard Operating Procedures, Rev No. 4.0, Feb 02, 2017. Both contract Labs have performed microscopy services as part of previous ACT/Naval Research Lab fluorometer testing under a ballast water compliance monitoring study, and have undergone previous Technical Audits by ACT's Quality Assurance Manager and both maintain rigorous protocols and certifications.

### *Colored Dissolved Organic Matter (CDOM)*

Approximately 40 ml of sample filtrate was used to rinse the collection flask and the 50 ml BD Falcon centrifuge tubes, and then discarded. Following the rinse, an additional 45 mls of the CDOM designated sample was filtered using 47 mm GF/F filters (0.7 µm pore size) with low vacuum pressure (<5 in Hg). The filtrate was captured in the centrifuge tube, capped, wrapped with Parafilm, labeled, and stored in a refrigerator (4° C) until analysis. All samples were shipped to MLML on dry ice for analysis using a calibrated laboratory-grade spectrophotometer. The sample and MilliQ blank were equilibrated to room temperature and spectrophotometric scans were run between 250 and 800 nm at 1 nm intervals, with a 4-5 nm slit width. Absorption from optical density was calculated by subtracting the optical density at 750 nm to correct for residual scattering and reported as the absorption at wavelength 400.

### *Turbidity*

Turbidity was measured on gently mixed raw water samples using a Hach 2100AN Turbidimeter, calibrated with certified turbimetric standards (Hach). In addition, continuous *in situ* turbidity measurements were generated during all testing with a calibrated EXO2 sonde.

### *Ancillary Data*

In conjunction with each water sample collection, ACT personnel recorded site-specific conditions from nearby river and tide gauges, meteorological stations, and visual observations of the water. Sampling information was logged on standardized datasheets and transmitted weekly to the ACT Chief Scientist for data archiving and QA/QC performance checks.

## Quality Management

All technical activities conducted by ACT comply with ACT's Quality Management System (QMS), which includes the policies, objectives, procedures, authority, and accountability needed to ensure quality in ACT's work processes, products, and services. The QMS provides the framework for quality assurance (QA) functions, which cover planning, implementation, and review of data collection activities and the use of data in decision-making, and quality control. The QMS also ensures that all ACT data collection and processing activities are carried out in a consistent manner, to produce data of known and documented quality that can be used with a high degree of certainty by the intended user to support specific decisions or actions regarding technology performance. ACT's QMS meets the requirements of ISO/IEC 17025:2005(E), *General requirements for the competence of testing and calibration laboratories*; the American National Standards Institute (ANSI)/American Society for Quality (ASQ) E4-2004 *Quality Systems for Environmental Data and Technology Programs*; and U.S. Environmental Protection Agency, quality standards for environmental data collection, production, and use.

## RESULTS OF LABORATORY TEST

Instrument accuracy of chlorophyll and phycocyanin determinations, and their resulting ratios, was evaluated in two separate laboratory tests which took place at Moss Landing Marine Laboratory (MLML) and the NOAA Great Lakes Environmental Research Laboratory (GLERL). In both cases, the tests involved a series of short-term exposures to various cultured phytoplankton species along with add-in matrix challenges for turbidity and CDOM.

### Moss Landing Marine Lab

Four lab tests with cultured algae were conducted from June 26 – June 29, 2017. Each test was conducted over the course of one day and involved a series of individual 50 – 60 minute trials. The test conditions for each individual trial are defined in tables 1 – 5, immediately preceding the presentation of results for that day. For the June 26 lab test, trials were conducted on individual freshwater and saltwater algal species made up in discrete 3 L batches. Prior to the algal exposures, background readings were taken on DI and the freshwater or saltwater culture media. Three freshwater algal culture trials were conducted using two different levels of *Coelastrum* additions (ca. 10 and 20 µg/L CHL) and one level of *Microcystis* (ca. 25 µg/L CHL and 1 µg/L PC) (Table 1).

Table 1. Test conditions for each trial of the June 26 MLML laboratory tests. This lab test focused on instrument response to two different freshwater algal species, *Coelastrum* and *Microcystis*, as well as, responses to DI and the freshwater culture media. (n = number of reference samples taken during the exposure; C1 and C2 refer to increasing concentration level from additional culture addition).

| Trial | *Coelastrum* | *Microcystis* |
|---|---|---|
| DI (n = 1) | - | - |
| FW Media (n = 2) | - | - |
| FW T1 (n = 2) | C1 | - |
| FW T2 (n = 2) | C2 | - |
| FW T3 (n = 2) | - | C1 |

Two reference samples were collected from each sample including one immediately after sample preparation and the second at the end of all instrument exposures (approximately 45 minutes later). However, for the CyanoFluor, exposure was done only from the initial sample split, instrument results are shown as the average and standard deviation of triplicate reads from that sample. A time constraint prevented us getting instrument reads on the final sample during this test. Results are plotted as a time series of instrument readings recorded in relative fluorescent readings compared to extracted pigment concentrations in µg/L determined on the reference samples (Figures 1). It should be noted for the bottom panel showing results for the PC/CHL ratio, no values are reported for DI or freshwater media exposure since these were blanks and the ratios are meaningless.

For the CHL channel, the CyanoFluor showed low background response and consistently tracked chorophyll levels for the two concentrations of *Coelastrum* and the one concentration of *Microcystis.* For the PC channel, the CyanFluor showed a high background signal to the DI and media exposures relative to the lowest concentration of *Coelastrum* (FW T1).

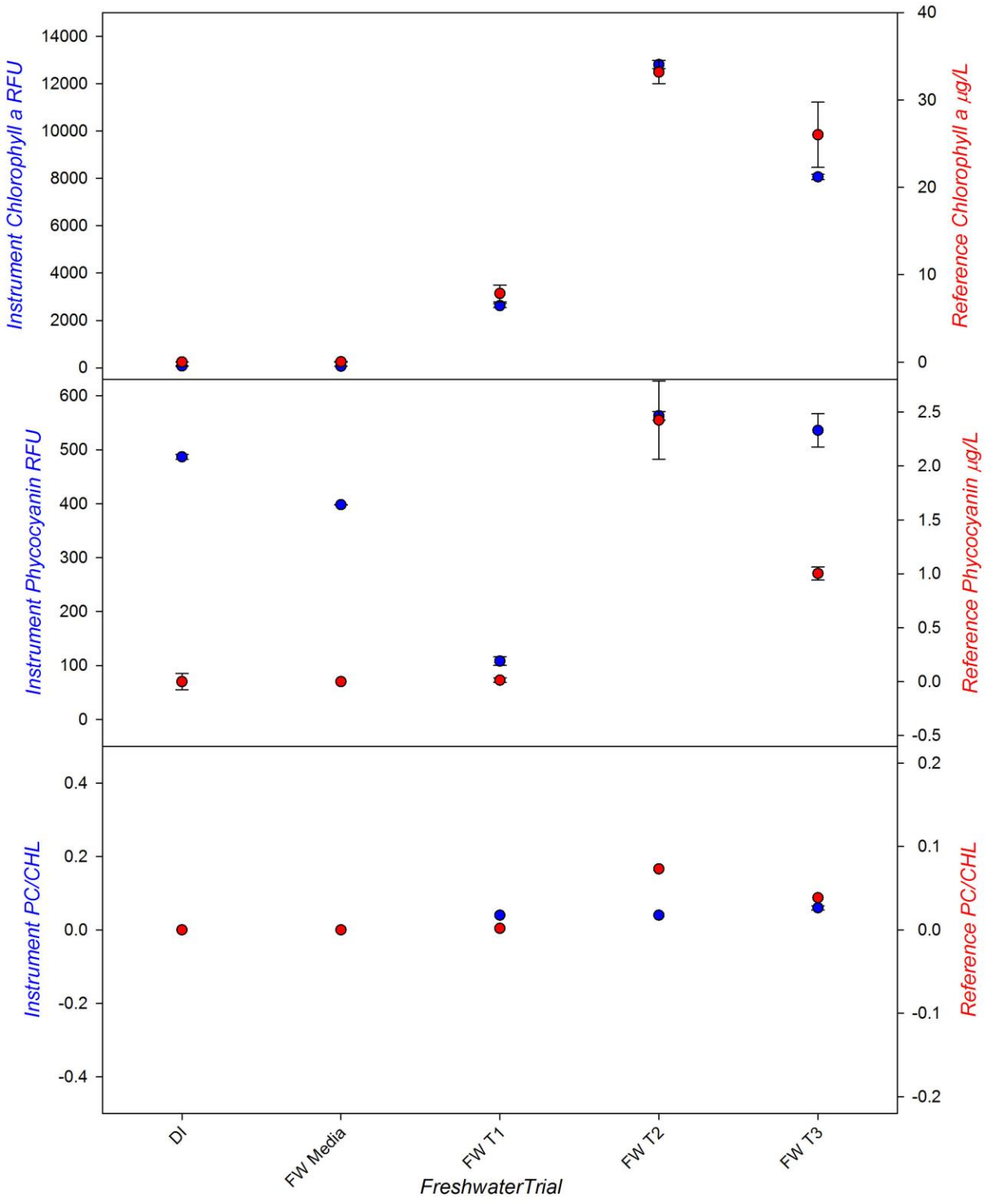



Figure 1. *Top Panel:* Plot of instrument (blue) and reference (red) measurements of chlorophyll a in the freshwater individual algae tests, including background readings for DI and the freshwater culture media. Two replicate reference measurements were made at each level while instrument reads were only read at the beginning of each trial. *Middle Panel:* Plot of measurements of phycocyanin. *Bottom Panel:* Plot of the ratio of phycocyanin/chlorophyll a.

Unexpectedly, the PC reads for the second addition of *Coelastrum* (FW T2) was about the same as for the exposure of the *Microcystis* culture (FW T3). The fact that the reference PC value for FW T2 was also higher indicates that there was some contamination present in the *Coelastrum* culture used in trial 1. There was a measureable PC response by the CyanoFluor to the pure *Microcystis* culture even though the sample concentration was quite low (1 µg/L). A cross plot of CyanoFluor readings compared to reference sample measurements for CHL is shown in figure 2. The regression line for the CHL response was highly significant (p=0.01) with an $R^2$ of 0.98 and a slope of 364 RFU/ µg/L. The regression for PC is shown but was not significant and not meaningful given the exceptionally small range of values and the relatively high background readings to the media blank. Therefore, no plot was made for the PC/CHL ratio.

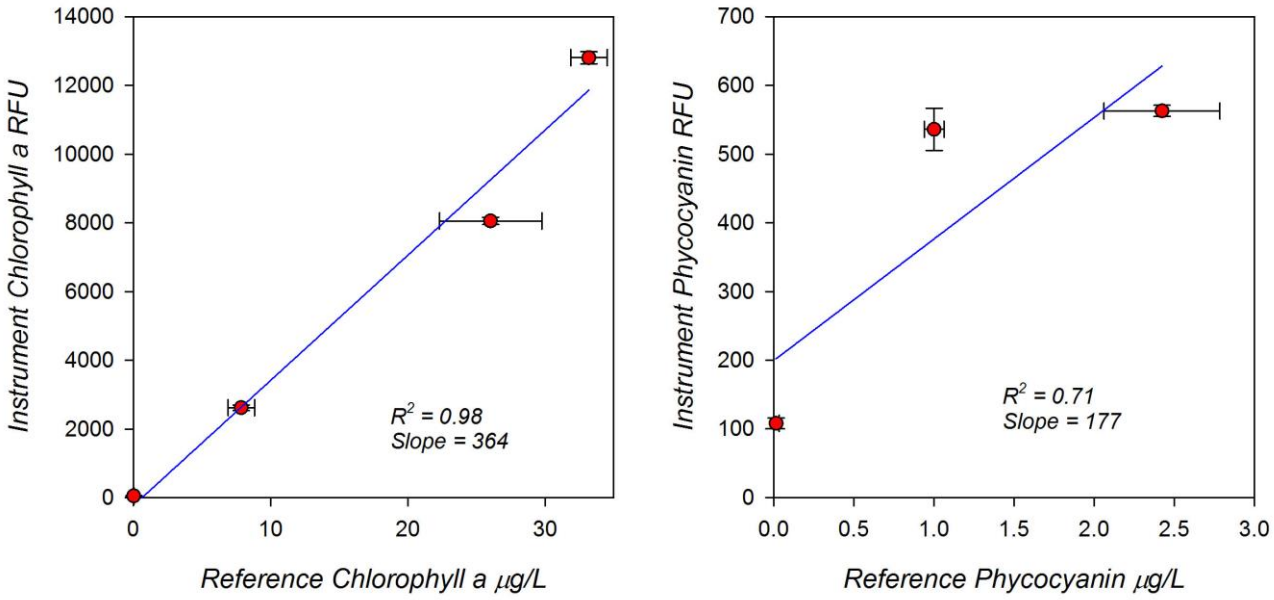



Figure 2. Cross plot of instrument readings versus extracted reference sample pigments for chlorophyll a and phycocyanin during the freshwater individual algae lab trials.

Four individual trials were conducted using saltwater algal cultures along with the DI and saltwater media blanks (Table 2). Trials 1 and 2 used two different levels of the golden-brown haptophyte *Isochrysis* at approximately 5 and 22 µg/L CHL and trials 3 and 4 used two different levels of the dinophylagellate *Amphidinium* added at concentrations of approximately 5 and 20 µg/L CHL. This culture is denoted as 'AC Mix' because microscopic examination indicated it was contaminated with other golden-brown diatoms that likely broke through the seawater filtration system.

Table 2. Description of test conditions for the individual saltwater algal culture exposures for the June 26 MLML laboratory tests. The test examined instrument response to two different saltwater algal species, *Isochrysis* and *Amphidinium* (denoted as 'AC Mix' because it was not a pure culture) along with background readings of DI and the saltwater culture media. (n = number of reference samples collected during the trial; C1 and C2 refer to increasing concentrations from additional culture addition).

| Trial | *Isochrysis* | AC Mix |
|---|---|---|
| DI (n = 1) | - | - |
| SW Media (n = 2) | - | - |
| SW T1 (n = 2) | C1 | - |
| SW T2 (n = 2) | C2 | - |
| SW T3 (n = 2) | - | C1 |
| SW T4 (n = 2) | - | C2 |

Results are plotted as a time series of instrument readings recorded in relative fluorescent readings compared to extracted pigment concentrations in µg/L determined on the reference samples (Figures 3). For the CHL channel, the CyanoFluor showed low background response and tracked chorophyll levels for the two concentrations of *Isochrysis* but only accurately responded to the first of the two AC mix exposures (top panel). There was no measureable PC in the reference samples and the CyanoFluor exhibited background readings ranging from 100 to 500 RFU. The bottom panel depicts results for the PC/CHL ratio but the values are not meaningful since there was no PC containing species used for these trials.

A cross plot of the CyanoFluor readings compared to reference sample measurements for CHL during the saltwater trials is shown in figure 4. The regression line for the CHL response was highly significant ($p=0.0001$) with an $R^2$ of 0.99 and a slope of 452 RFU/ µg/L. Regressions on comparative measurements for PC and PC/CHL ratio were not computed.

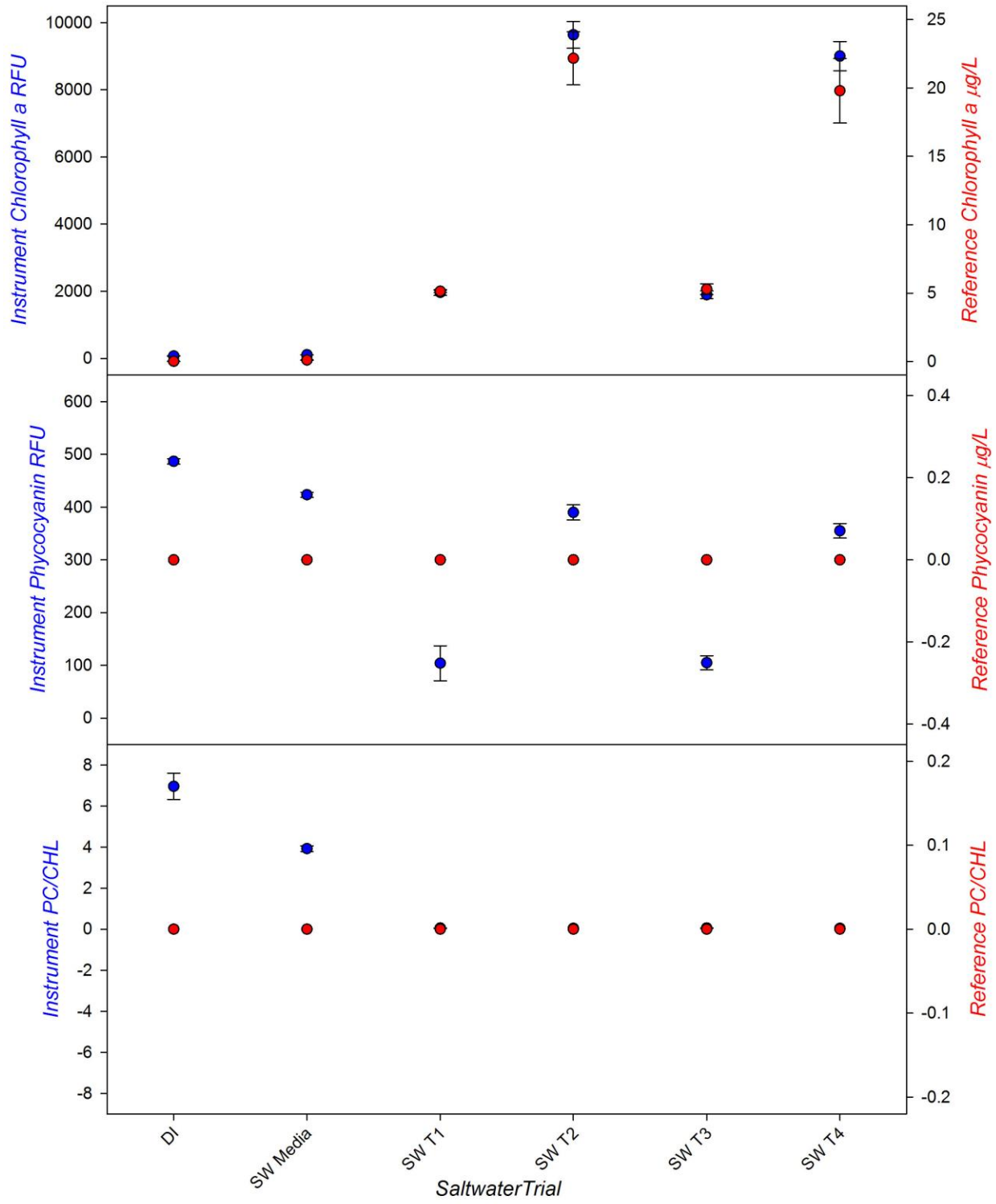



Figure 3. *Top Panel:* Plot of instrument (blue) and reference (red) measurements of chlorophyll a in the saltwater individual algae tests including background readings for DI and saltwater media. A single reference sample was taken just prior to exposure and the CyanoFluor measurements represent the average of triplicate reads for each sample. *Middle Panel:* Plot of measurements of phycocyanin. *Bottom Panel:* Plot of the ratio of phycocyanin/chlorophyll a.

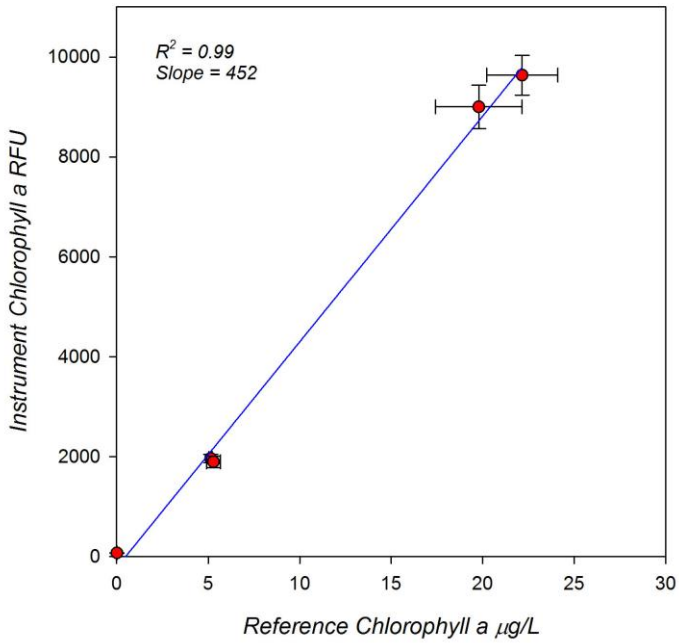



Figure 4. Cross plot of instrument readings versus extracted reference sample pigments for chlorophyll a during the saltwater individual algae lab trials.

On June 27th, 11 different trials were conducted using four levels of *Synechococcus* with add-in matrix challenges of three CDOM levels and two turbidity levels, plus background culture media (Table 3). Each test condition was made up in a 40 L container and reference samples were withdrawn at three timepoints over the course of a 30-minute exposure period. An aliquot for triplicate CyanoFluor readings was split from each reference sample draw. It must be noted that the *Synechococcus* culture became contaminated with large marine Diatoms which ended up dominating the community in terms of biovolume (98%) even though the numerical abundance of the small *Synechococcus* cells was greater.

Table 3. June 27 test conditions with exposures to combinations of a saltwater *Synechococcus* cultures at various concentrations with add-in challenges of turbidity and CDOM. (n denotes number of reference samples collected and the values in parenthesis show averaged concentrations determined on the reference samples)

| Trial | *Synechococcus* (CHL µg/L) | CDOM ($A_{400}$) | Turbidity (NTU) |
|---|---|---|---|
| SW Media (n = 1) | - | - (0.25) | - (0.69) |
| SW T2 (n = 3) | C1 (3.2) | - (0.25) | - (0.55) |
| SW T3 (n = 3) | C2 (6.4) | - (0.24) | - (0.54) |
| SW T4 (n = 3) | C2 (6.1) | C1 (0.83) | - (0.34) |
| SW T5 (n = 3) | C2 (6.2) | C2 (1.76) | - (0.48) |
| SW T6 (n = 3) | C2 (6.0) | C3 (3.25) | - (0.44) |
| SW T7 (n = 3) | C3 (19) | C3 (3.05) | - (0.65) |
| SW T8 (n = 3) | C4 (38) | C3 (2.86) | - (1.0) |
| SW T9 (n = 3) | C4 (40) | C3 (2.90) | C1 (3.3) |
| SW T10 (n = 3) | C4 (37) | C3 (2.90) | C2 (23) |
| SW T11 (n = 3) | C4 (36) | C3 (2.97) | C3 (50) |

Over the ten algal trials, CHL levels ranged from 3.2 to 40 µg/L and PC levels ranged from 0 to 1.5 µg/L for the reference samples. CDOM additions increased from background levels of approximately 0.25 up to 3.0, and turbidity additions increased levels from a background of 0.5 up to 50 NTU.

Results are plotted as a time series of instrument readings recorded in relative fluorescent units compared to extracted pigment concentrations in µg/L determined on the reference samples (Figures 5). For the CHL channel, the CyanoFluor showed low background response and tracked chorophyll levels closely across the whole range of concentrations, however, the offset from reference measurements did increase at the two highest turbidity levels (Trials 10 and 11). The CyanoFluor PC readings had a much lower background reading in the media than for previous tests and tracked reference measurements closely across all trials, but with somewhat greater offset in two of the three timepoints for trial 10. However, this offset could not necessarily be attributed to the turbidity addition since the doubling of turbidity in trial 11 did not show a similar response. Again, the PC levels were quite low and this amount of variability is not highly meaningful. Similarly the variability in the PC/CHL ratio is not meaningful given these concentration levels.

A cross plot of the CyanoFluor readings compared to reference sample measurements for CHL during the saltwater trials is shown in figure 6. The regression line for the CHL response was highly significant ($p<0.001$) with an $R^2$ of 0.96 and a slope of 156 RFU/ µg/L. This response slope is noticeably lower than for the previous two Lab tests. The regression line for the PC response was also highly significant ($p<0.001$) with an $R^2$ of 0.92 and a slope of 308 RFU/ µg/L even across this very small range of concentrations. Background readings to the SW media were much lower than for the previous tests. The response slope is noticeably lower than for the previous two Lab tests. The regression of comparative PC/CHL estimations was not computed given the low values and high variability.

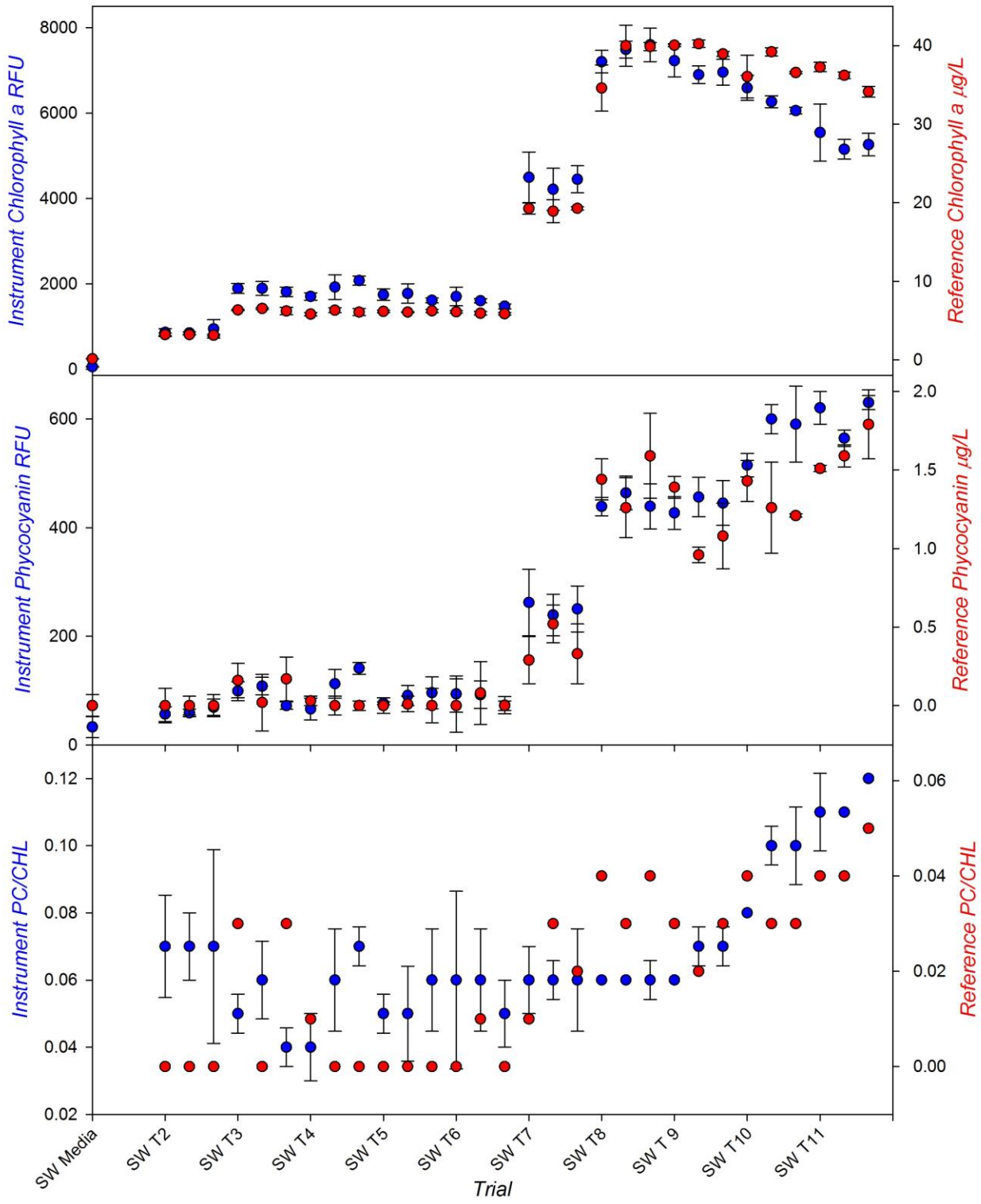



Figure 5. *Top Panel:* Plot of instrument (blue) and reference (red) measurements of chlorophyll a in the saltwater CDOM and turbidity addition trials covering 4 algae, 3 CDOM and 3 turbidity concentration levels. Three replicate reference measurements were made at each level with only one read in blank media, each sample was read in triplicate on the CyanoFluor. *Middle Panel:* Plot of measurements of phycocyanin. *Bottom Panel:* Plot of the ratio of phycocyanin/chlorophyll a.

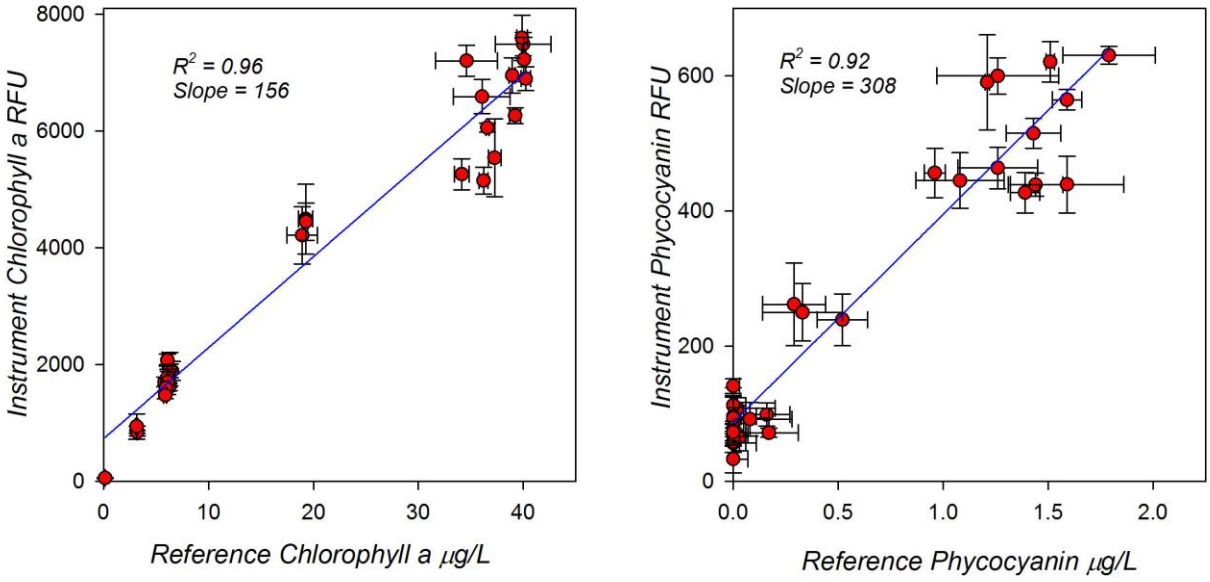



Figure 6. Cross plot of instrument readings versus extracted reference sample pigments for chlorophyll a and phycocyanin during the saltwater *Synechococcus* algae exposure with add in matrix challenges for CDOM and Turbdity.

On June 28th, 7 different trials were conducted covering six algal concentrations and different mixtures using *Coelastrum*, *Microcystis* and *Peridinium* at varying concentrations, and a freshwater media blank (Table 4). Each test condition was made up in a 40 L container and reference samples were withdrawn at three timepoints over the course of a 30 minute exposure period. An aliquot for triplicate CyanoFluor readings was split from each reference sample. Over the six algal trials, CHL levels ranged from 3.8 to 25 µg/L and PC levels ranged from 1.3 to 6.7 µg/L.

**Table 4.** June 28 test conditions with exposures to combinations of freshwater algal cultures at various concentrations. (n = number of reference samples taken during the exposure; C1 and C2 represent concentration levels from culture additions).

| Trial | *Coelastrum* | *Microcystis* | *Peridinium* |
|---|---|---|---|
| FW Media (n = 1) | - | - | - |
| FW T2 (n = 3) | C1 | - | - |
| FW T3 (n = 3) | C2 | - | - |
| FW T4 (n = 3) | C2 | C1 | - |
| FW T5 (n = 3) | C2 | C2 | - |
| FW T6 (n = 3) | C2 | C3 | - |
| FW T7 (n = 3) | C2 | C3 | C1 |

Results for the June 28 lab test with freshwater algal mixtures are plotted as a time series of instrument readings recorded in relative fluorescent units compared to extracted pigment concentrations in µg/L determined on the reference samples (Figures 7). For the CHL readings, the CyanoFluor showed low background response to the media and tracked chorophyll levels consistently over the tested range from 4 to 25 µg/L and across all mixtures of the three algal groups. For the PC measurements, there was an initial increase in readings from 50 to 200 RFU during trial 2 with the second addition of *Coelastrum* which was consistent with our reference value increasing from 0 to 1 µg/L phycocyanin, and as noted previously indicated some cyanobacterial contamination in our *Coelastrum* culture. The CyanoFluor closely tracked the three additions of the *Microcystis* culture through the top level of 6.7 µg/L with a corresponding RFU reading of approximately 650. The computed PC/CHL ratios for the CyanoFluor were very low and did not consistently track the ratios computed for the reference samples. The CyanoFluor values mostly averaged around 0.05 across all trials, compared to the range in reference data from 0.02 to 0.23. However, it should be noted that the test covered a very small range of PC levels relative to what would be found in cyanobacterial blooms in the natural environment (see Field Results).

Cross plots of the CyanoFluor readings compared to reference sample measurements for CHL and PC during the freshwater algal mixture trials are shown in figure 8. The regression line for the CHL response was highly significant ($p<0.001$) with an $R^2$ of 0.99 and a slope of 417 RFU/ µg/L. The regression line for the PC response was also highly significant ($p<0.001$) with an $R^2$ of 0.95 and a slope of 84.8 RFU/ µg/L. A cross plot of the CyanoFluor readings compared to reference sample measurements for the PC:CHL ratio was not quite significant ($p=0.09$) with an $R^2$ of 0.17 and the near-zero slope of 0.046 for this small range.

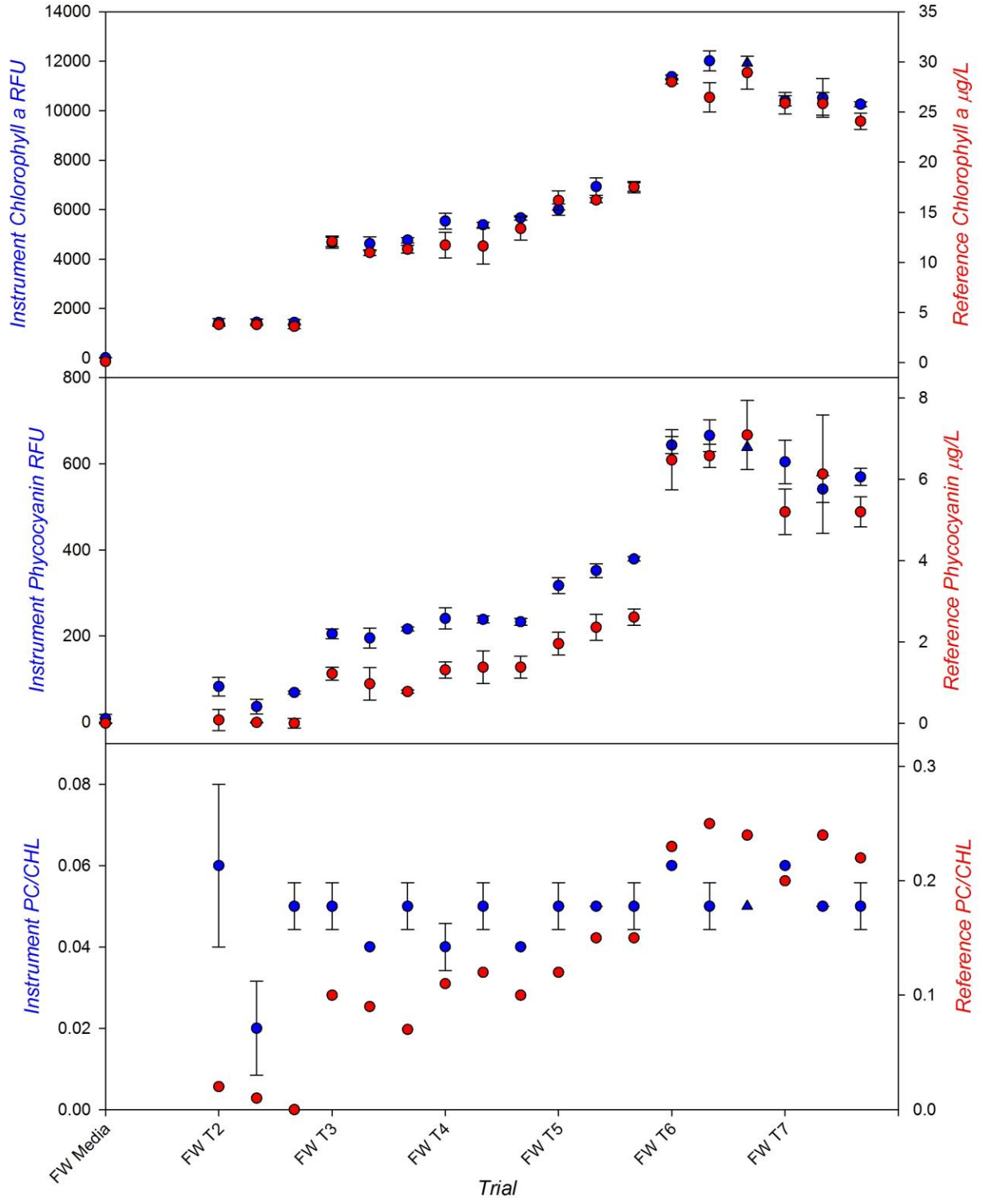



Figure 7. *Top Panel:* Plot of instrument (blue) and reference (red) measurements of chlorophyll a in the freshwater algal mixture trials covering 6 concentration ranges and mixtures of 3 different algae. Three replicate reference measurements were made at each level, each sample was read in triplicate on the CyanoFluor. Triangular data points represent Cyano instrument reads of less than 3 due to omission of bad data. *Middle Panel:* Plot of measurements of phycocyanin. *Bottom Panel:* Plot of the ratio of phycocyanin/chlorophyll a.

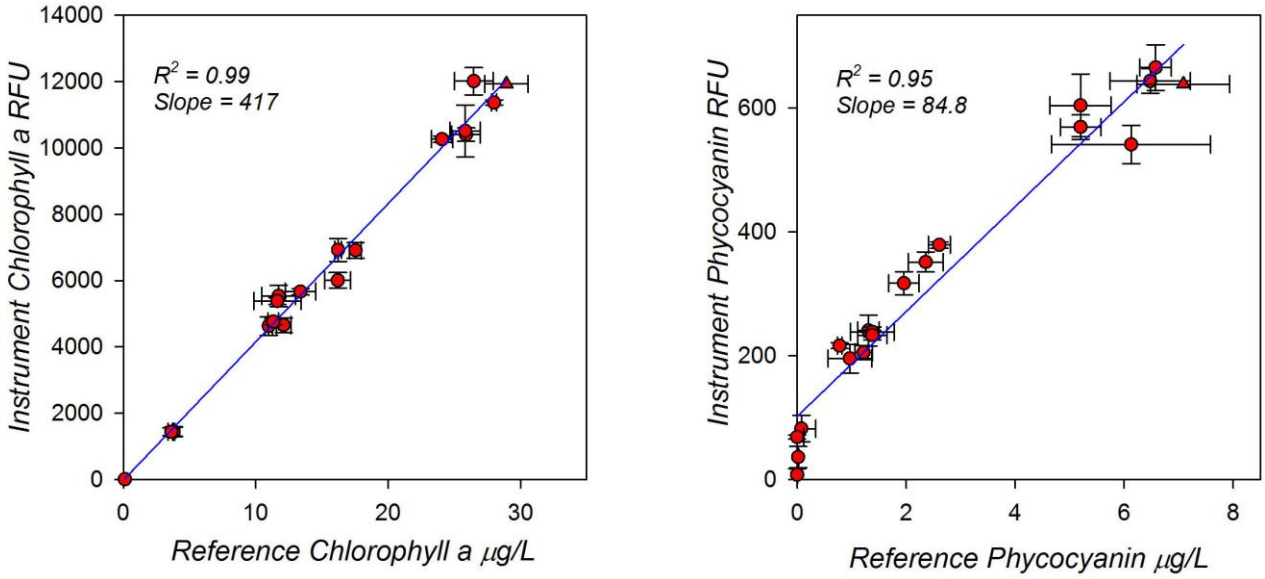



Figure 8. Cross plot of instrument readings versus extracted reference sample pigments for chlorophyll a (*left*) and phycocyanin (*right*) during the June 28 freshwater algal mixtures lab trials. The blue lines represent the linear regression of the data.

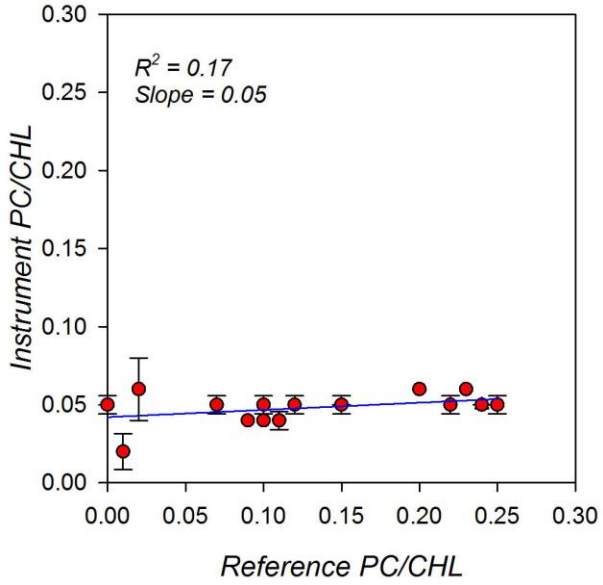



Figure 9. Cross plot of instrument readings versus extracted reference sample pigments for the PC:CHL ratio during the June 28 freshwater algal mixtures lab trials. The blue lines represent the linear regression of the data..

For the June 29 lab test, nine different trials (plus background media) were conducted using three levels of *Microcystis* with add-in matrix challenges of three CDOM levels, followed by an addition of *Coelastrum*, and lastly an addition of turbidity (Table 5). Each test condition was made up in a 40 L container and reference samples were withdrawn at two or three timepoints over the course of a 30-minute exposure period. An aliquot for triplicate CyanoFluor readings was split from each reference sample. Over the eight trials, CHL levels ranged from 5.3 to 59 µg/L and PC levels ranged from 0.2 to 9.8 µg/L. CDOM additions increased concentrations from a background of 0.43 up to 4.7 and the turbidity additions increased concentrations from a background of 1 up to 25 NTU.

Table 5. June 29 test conditions with exposures to combinations of freshwater algal cultures at various concentrations with add-in challenges of turbidity and CDOM. Designations with C#, indicate additions, or concentration levels of the specific parameter. Measured concentrations of CDOM and Turbidity for reference samples are provided in parenthesis.

| Trial | *Microcystis* | CDOM | *Coelastrum* | Turbidity |
|---|---|---|---|---|
| FW Media (n = 1) | - | - | - | - |
| FW T2 (n = 3) | C1 | - (0.43) | - | - (0.25) |
| FW T3 (n = 3) | C2 | - (0.43) | - | - (0.39) |
| FW T4 (n = 3) | C2 | C1 (1.5) | - | - (0.38) |
| FW T5 (n = 3) | C2 | C2 (3.3) | - | - (0.45) |
| FW T6 (n = 2) | C2 | C3 (6.4) | - | - (0.56) |
| FW T7 (n = 2) | C3 | C3 (4.6) | - | - (0.86) |
| FW T8 (n = 2) | C3 | C3 (4.6) | C1 | - (1.1) |
| FW T9 (n = 2) | C3 | C3 (4.7) | C1 | C1 (25) |

Results for the June 29 lab test with freshwater algal mixtures and CDOM and turbidity additions are plotted as a time series of instrument readings in RFU compared to reference sample pigment concentrations in µg/L (Figures 10). For CHL readings, the CyanoFluor showed low background response to the media (51 RFU) and tracked chorophyll levels consistently over the test range from 5 to 59 µg/L across both algal groups and without any noticeable impacts from the CDOM and turbidity challenges. There was significant variation in the two reference sample CHL estimates for trial 8 (grey symbol), but not for the CyanoFluor. Cell counts in replicate two of the reference samples were also 25% lower suggesting some patchiness in the tank during the sub-sampling. For PC measurements, the background response to the media was significantly lower than seen for other lab tests (30 RFU) and the response tracked all *Microcystis* additions up to the peak addition of 9.8 µg/L. There was a significant mismatch for the final trial where turbidity was added, however, it was not expected that the PC concentration in the tank should have decreased more than a few percent by dilution of the turbidity addition, so the disagreement is likely a result of a bad reference sample (grey symbols) and NOT an inaccurate reading by the CyanoFluor. The computed PC/CHL ratios for the CyanoFluor did not match the observed pattern seen for the reference sample, and averaged around 0.07 across all trials, compared to the range in reference data from 0.02 to 0.26. Again, it should be noted that the test covered only a small range of PC levels relative to what would be found in cyanobacterial blooms in the natural environment (see Field Results).

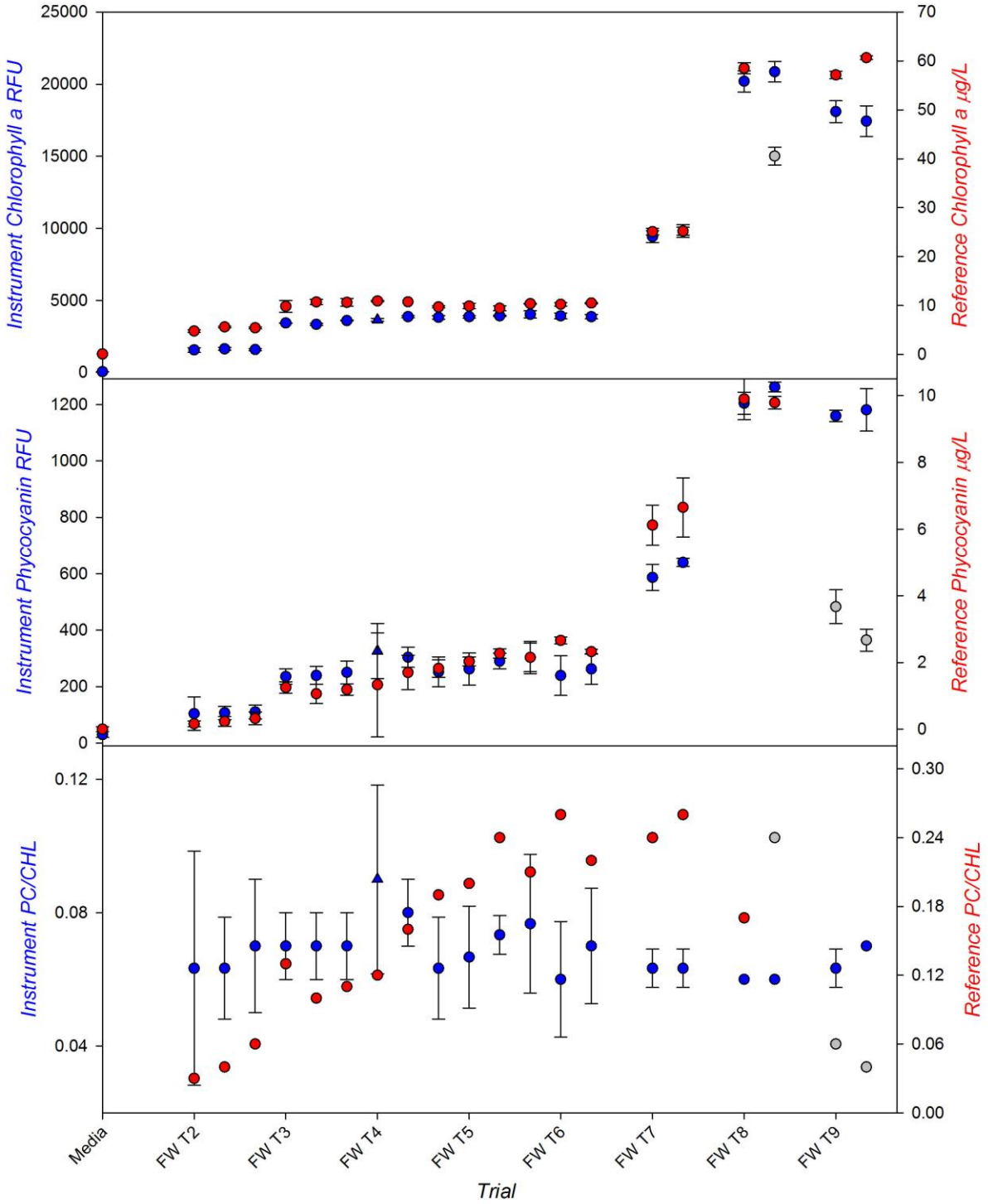



Figure 10. *Top Panel:* Plot of instrument (blue) and reference (red) measurements of chlorophyll a during the June 29 laboratory test with freshwater algae *Microcystis* and *Coelastrum*, and challenge additions of CDOM and turbidity. Three replicate reference measurements were made for trials 2-6, two replicates were made for trials 7-9, and one replicate for the blank media. Each reference sample was read in triplicate on the CyanoFluor. *Middle Panel:* Plot of measurements of phycocyanin. *Bottom Panel:* Plot of the ratio of phycocyanin/chlorophyll a.

Cross plots of the CyanoFluor readings compared to reference sample measurements for CHL and PC during the freshwater algal mixture trials are shown in figure 11. Suspect reference data (grey symbols in figure 10) are omitted from these analyses. The regression line for the CHL response was highly significant ($p<0.001$) with an $R^2$ of 0.98 and a slope of 313 RFU/ µg/L. The regression line for the PC response was also highly significant ($p<0.001$) with an $R^2$ of 0.95 and a slope of 108 RFU/ µg/L.

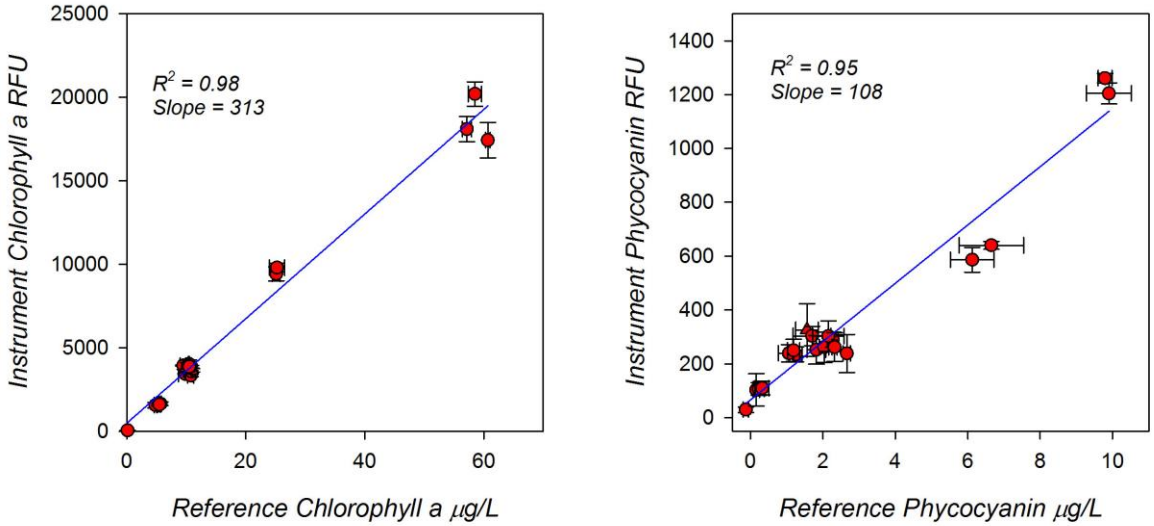
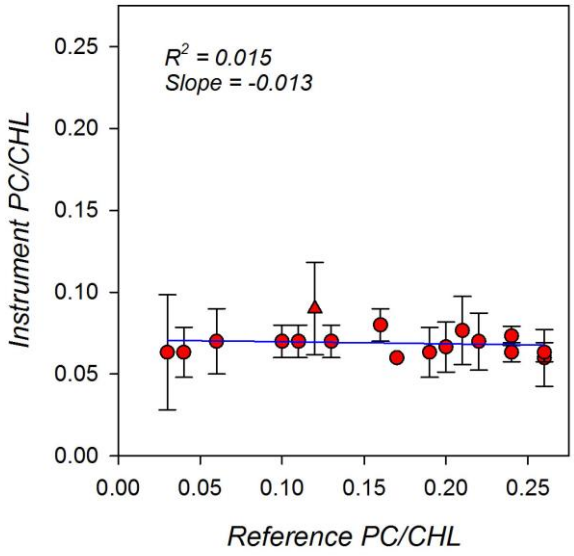



Figure 11. Response plot for the CyanoFluor estimations of chlorophyll a (*left*) and phycocyanin (*right*) during exposures to combinations of freshwater algal cultures at various concentrations with add-in challenges of turbidity and CDOM compared to extracted concentrations measured on reference samples. The blue lines represent the linear regression of the data.




A cross plot of the CyanoFluor estimates of the PC/CHL ratio compared to the ratio computed for extracted concentrations measured on the reference samples was not significant ($p=0.64$) with an $R^2$ of 0.15 and a small negative slope of- 0.013.

Figure 12. Response plot for CyanoFluor estimation of the PC/CHL ratio compared to ratio of extracted PC and CHL from reference samples during exposures to combinations of freshwater algal cultures at various concentrations with add-in challenges of turbidity and CDOM .

**Great Lakes Freshwater Lab Test**

A second laboratory test with freshwater algal cultures was conducted at the NOAA Great Lakes Environmental Research Lab (GLERL) on August 10, 2017. This test was added after the development of the original test protocols to help address the contamination that occurred in the MLML lab cultures and to test at higher levels of cyanobacteria and PC. The GLERL freshwater lab test was conducted over the course of one day and involved a series of nine individual trials (Table 6). The first four trials involved exposures to single algae monocultures of *Chlorella*, *Cryptomonas*, *Aphanizomenon*, and *Peridinium* at concentrations levels defined in Table 6. The next four trials used a mixture of all four algal species to assess the ability to discriminate among the distinct algal taxa when present in different ratios. The last trial incorporated challenge additions of both CDOM and turbidity to the same algal composition as the proceeding trial. Each test solution was made up in discrete 2 L batches by combining known quantities of the cultures into a fixed volume of freshwater media. Triplicate readings of each test solution were conducted on the CyanoFluor.

Table 6. Great Lakes lab test conducted on August 10th with exposures to four individual freshwater algal cultures followed by four mixtures of all 4 algal species at various ratios, followed by an add-in challenge of turbidity and CDOM. For the mixtures, a capital letter denotes abundance at the higher C2 level and a lower case letter denotes the C1 abundance level that was 20% of C2. Reference sample CHL concentrations in µg/L for each culture addition (directly measured for monocultures and based on volumetric addition for mixtures) are provided in parenthesis.

| Trial | *Chlorella* (µg/L) | *Cryptomonas* (µg/L) | *Aphanizomenon* (µg/L) | *Peridinium* (µg/L) | CDOM ($A_{400}$) | Turbidity (NTU) |
|---|---|---|---|---|---|---|
| A | C2 (12.1) | - | - | - | - | - |
| B | - | C2 (12.7) | - | - | - | - |
| C | - | - | C2 (28.6) | - | - | - |
| D | - | - | - | C2 (12.4) | - | - |
| Abcd | C2 (12.1) | C1 (2.5) | C1 (5.7) | C1 (2.5) | - | - |
| ABcd | C2 (12.1) | C2 (12.7) | C1 (5.7) | C1 (2.5) | - | - |
| ABCd | C2 (12.1) | C2 (12.7) | C2 (28.6) | C1 (2.5) | - | - |
| ABCD | C2 (12.1) | C2 (12.7) | C2 (28.6) | C2 (12.4) | - | - |
| ABCD +Turb+CDOM | C2 (12.1) | C2 (12.7) | C2 (28.6) | C2 (12.4) | C1 (6.0) | C1 (33) |

Results are plotted as a time series of instrument measurements in RFU compared to extracted pigment concentrations in µg/L determined on the reference samples (Figures 13). Overall, the CyanoFluor tracked the CHL concentration over the tested range from 12 to 66 µg/L, however, it over-estimated CHL concentrations for the *Cryptomonas* culture and under-estimated concentrations for the *Aphanizomenon* culture. The response to the mixtures was therefore dependent on the proportions of these species. Individual response curves to the monocultures were not established to accurately quantify these biases. Overall, the CyanoFluor tracked the PC concentration over the tested range from 0.2 to 65 µg/L. There was no known reason for the false high PC reading for *Chlorella* (Trial A), and this response was not seen again in any of the mixtures that included *Chlorella.* The CyanoFluor PC measurement for *Cryptomonas* was also higher than expected but again not seen when added in mixtures. The reference PC determination in the last trial with CDOM and turbidity was unexpectedly high and should have more closely matched the previous two trials as was observed in the CyanoFluor determinations. The

CyanoFluor PC:CHL ratios did not scale consistently with ratios for the reference samples.

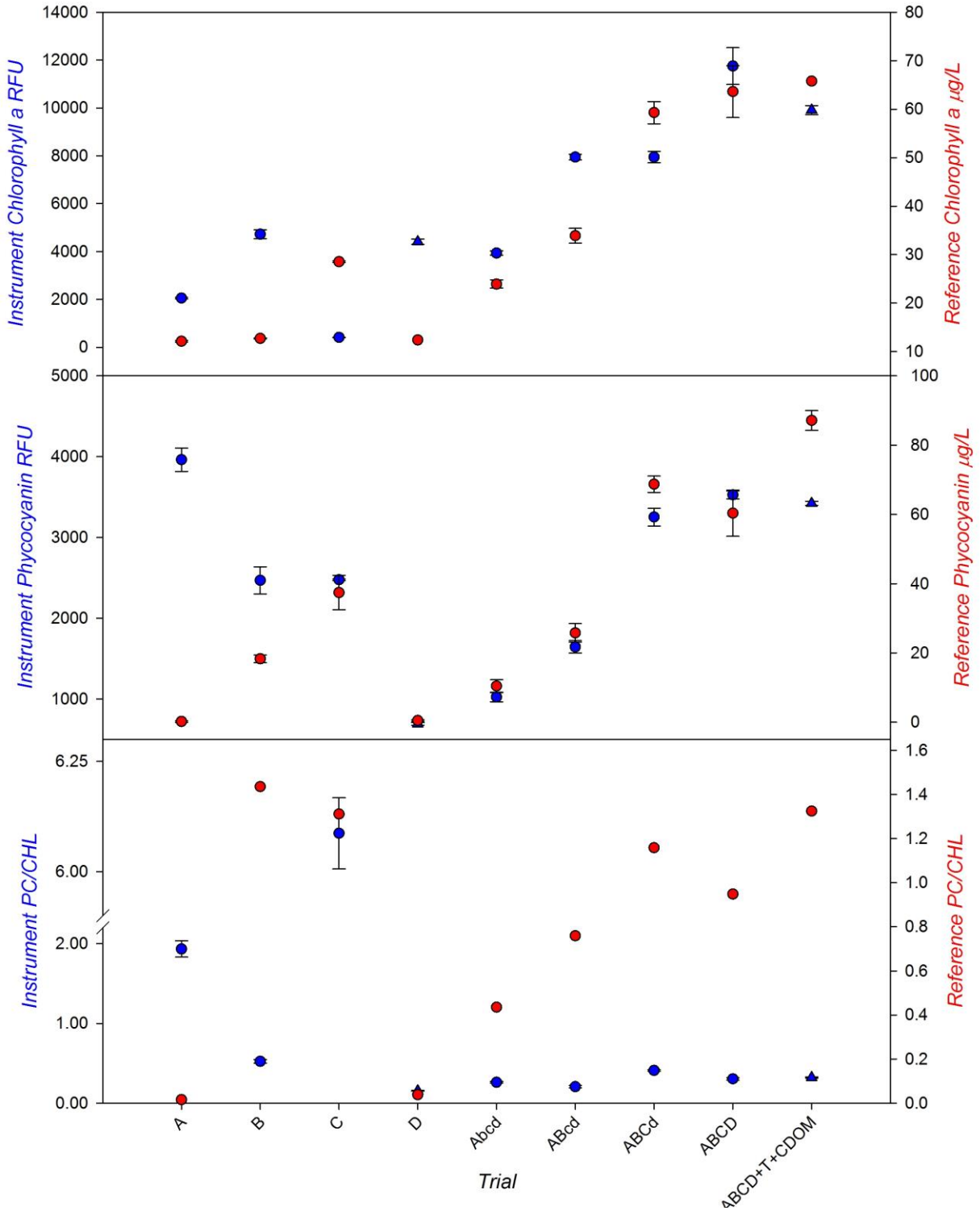



Figure 13. *Top Panel:* Plot of instrument (blue) and reference (red) measurements of chlorophyll a in the Great Lakes laboratory trial over 4 individual algae cultures, 4 mixtures and a CDOM and turbidity addition. One reference measurement was made at each level while each sample was read in triplicate on the CyanoFluor, the triangles represent a 2 point average due to the omission of a bad measurement. *Middle Panel:* Plot of measurements of phycocyanin. *Bottom Panel:* Plot of the ratio of phycocyanin/chlorophyll a.

One-to-one cross plots of the CyanoFluor readings compared to reference sample measurements for CHL and PC during the freshwater algal mixture trials are shown in figure 14. The regression line for the CHL response was highly significant (p=0.008) with an $R^2$ of 0.66 and a slope of 134 RFU/ µg/L. The regression line for the PC response using all orginial data was not significant (p=0.11) with an $R^2$ of 0.32 and a slope of 21.2 RFU/ µg/L. If the false high CyanoFluor reading from Trial A is eliminated (as plotted in Fig. 14, right panel), then the regression was highly significant (p=0.002) with an $R^2$ of 0.82 and a slope of 32.3 RFU/ µg/L.

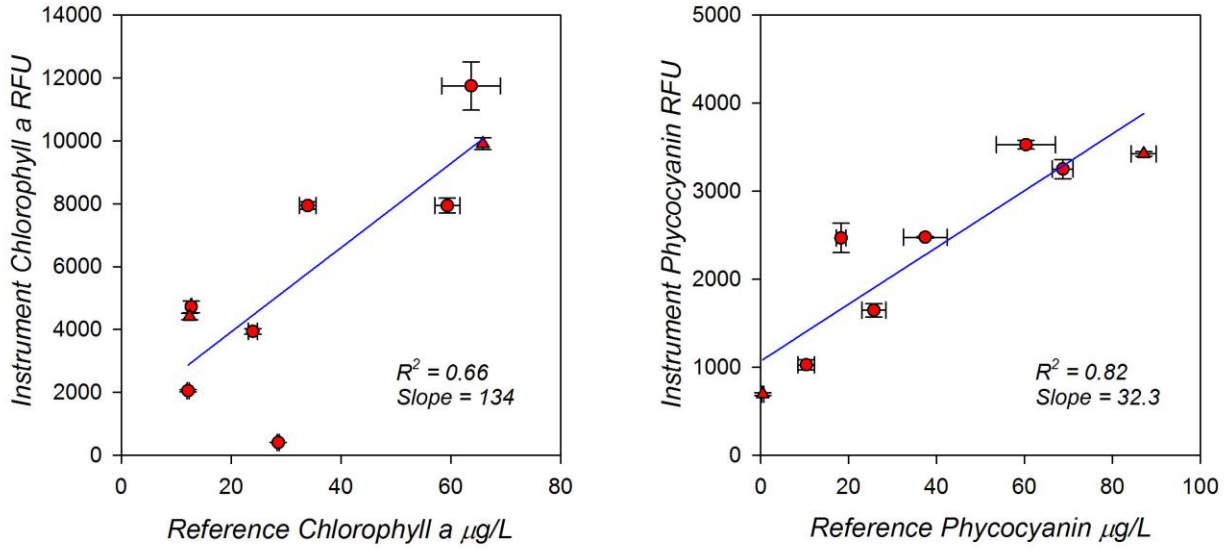
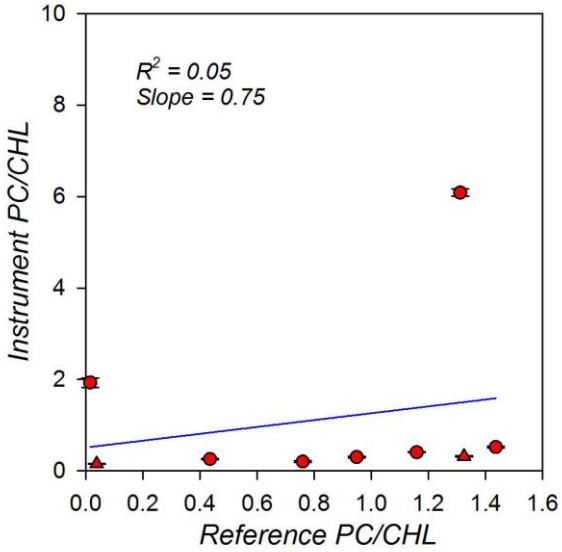



Figure 14. Response plot for the GL lab trial of the CyanoFluor chlorophyll a (left) and phycocyanin (right) compared to reference samples. The instrument values were obtained by averaging three instrument reads. The triangles represent 2-point averages due to the omission of an outlying reading. The blue lines represent the linear regression of the data.




A cross plot of the CyanoFluor readings compared to reference sample measurements for the PC:CHL ratio (Figure 15) was not significant (p=0.58) with an $R^2$ of 0.05 and a slope of 0.75.

Figure 15. Response plot for the GL lab trial of the CyanoFluor ratio of phycocyanin/chlorophyll compared to the extracted phycocyanin/chlorophyll ratio from matched reference samples.

FIELD TESTS

Five field tests were conducted as part of the performance evaluation of the CyanoFluor including three underway surface mapping applications and two mooring applications. The three surface mapping applications were conducted in San Francisco Bay, Monterey Bay, and western Lake Erie. The two moored deployment tests were conducted in the Maumee River, Waterville, OH and in Chesapeake Bay, Solomons Island, MD. Further descriptions of each test are provided below. Before the beginning of field testing at each site, the local ACT Partner performed a reference dye test using two concentrations of a commonly prepared BB3 dye from MLML, and a DI reading. The dye readings were done to check the working order of the CyanoFluor and the consistency of its response over the time course of the evaluation.

Table 7. Results of the pre-deployment BB3 dye check for the CyanoFluor for each deployment site. (n.d. denotes no data for that observation.)

| Date | Deployment Site | DI | BB3 0.05 µM | BB3 0.50 µM |
|---|---|---|---|---|
| **6/25/17** | **MLML** | 0.0 ± 0.0 | 42.0 ± 0.28 | 351.2 ± 0.71 |
| **8/10/17** | **UM** | 0.01 ± 0.01 | 38.1 ± 0.16 | 322.3 ± 0.51 |
| **9/05/17** | **CBL** | n.d. | 38.3 ± 0.31 | 323.3 ± 0.31 |

### *Surface Mapping Applications*

#### San Francisco Bay, CA

*R/V David H. Peterson*

USGS Menlo Park has conducted monthly water quality surveys along the axis of South San Francisco Bay, through the central bay, San Pablo and Susuin Bay and into the Sacramento delta since 1968 (https://sfbay.wr.usgs.gov/access/wqdata/index.html). This historical and ongoing set of observations has revealed tremendous plankton diversity along the transect ranging from protistan grazer dominated communities in the shallow warmer South Bay, to oceanic influenced communities in the Central Bay through the Golden Gate, and freshwater influenced communities eastward through the northern bays and Sacramento River. ACT's ongoing collaboration with USGS enabled us to leverage their transect design and research platform for a dedicated surface mapping cruise on 6 July 2017 onboard the *R/V David H. Peterson* (*photo right*). The cruise departed from the berth at the Redwood City Yacht Club on Redwood Creek, north along the axis of the South Bay, transited north to the Golden Gate Bridge in the west Central Bay, north into San Pablo Bay and returned southward below Redwood Creek to sample the shallow, warm and lower salinity waters of the southern reach of the South Bay before returning to dock (Figure 16). During the 150km underway mapping cruise eight stations were selected to make comparative reference sample measurements.

The USGS survey vessel, is equipped with a flow-through seawater system powered by a Headhunter Stingray continuous flow pump (20GPM) drawing from a through hull port at

approximately 1 m depth near the bow. A 40 gal black polyethylene trash can was plumbed with one-inch PVC inflow ports 1 inch from the bottom and 4 inches below the top and these were attached to valves which allowed us to control flow rates into the tank. Flow rate was sufficient to fill the exposure tank to the overflow port within 2 min. Instruments were hung from a 3 inch OD. PVC frame with sensors oriented toward the bottom of the tank. Coordinated rotation of the rack and intruments was used to clear instruments of accumulated bubbles and debris. Port valves were open during between station transit to permit continuous turnover of the contained water. The tank lid was keep closed except when sampling and to mix exposure water. Once on station, the inflow port valve was closed after 2 min and instruments were allowed to equilibrate for 10 min, then two reference samples were withdrawn at 10 and 20 minutes after isolation. Sampling was below the water surface near the sensor depth. An aliquot of the reference sample draw was used to expose the CyanFluor with three replicate measurements for each sample. After the 20-min sampling period, the tank was reopened to flow through while transiting to the next station.


A YSI EXO2 sonde in the tank provided continuous monitoring results during both underway and isolated time periods with measurements taken every 15 seconds (Figure 17). Continuous measurements indicated that conditions in the tank during isolation periods were relatively stable. Lab analysis of reference samples for CDOM and turbidity are plotted over the sonde data for consistency with other tests. During the San Francisco Bay cruise temperature ranged from 17 to 22 $^{o}$C and salinity ranged from 21.5 to 27.

Figure 16. Survey track for the underway surface mapping test in San Francisco Bay on the USGS *R/V Peterson*. Green triangles denote isolated, comparative sampling stations.

A time series of the CyanoFluor measurements of CHL, PC, and PC:CHL ratio are plotted against the corresponding reference measurements in figure 18. Extractable chlorophyll a, a proxy for total phytoplankton biomass, ranged from *ca*. 3 to 16 µg/L along the sampling transect with highest concentrations encountered in the southern end of South Bay (station 7). In contrast, extractable phycocyanin, a proxy for cyanobacterial biomass, was low throughout the survey, ranging only from 0 to 0.12 µg/L. The CyanoFluor CHL measurements (1050 – 2600 RFU) were congruent with their corresponding reference samples (Figure 18). A linear regression of the CyanoFluor chlorophyll RFU measurements against the extracted chlorophyll (Fig. 19) was highly significant ($p<0.001$) with an $R^2=0.96$ and a slope of 116 RFU/µg/L. CyanoFluor measures of PC fluorescence were low, ranging from 70 to 350 RFU, and consistent with the many non-detect or low extractable PC biomass in this ecosystem (Figure 18). As a consequence of the near-baseline PC levels, no significant correlation was obtained between *in vivo* PC fluorescence and extractable PC (Fig. 19). The CyanoFluor computed PC/CHL ratios (Fig. 18) were consistently low (ca 0.11) across the transect and higher than the ratio obtained by pigment extraction. As a consequence of low PC abundance and concentration range, there was no correlation between CyanoFluor and extractable PC/CHL estimates.

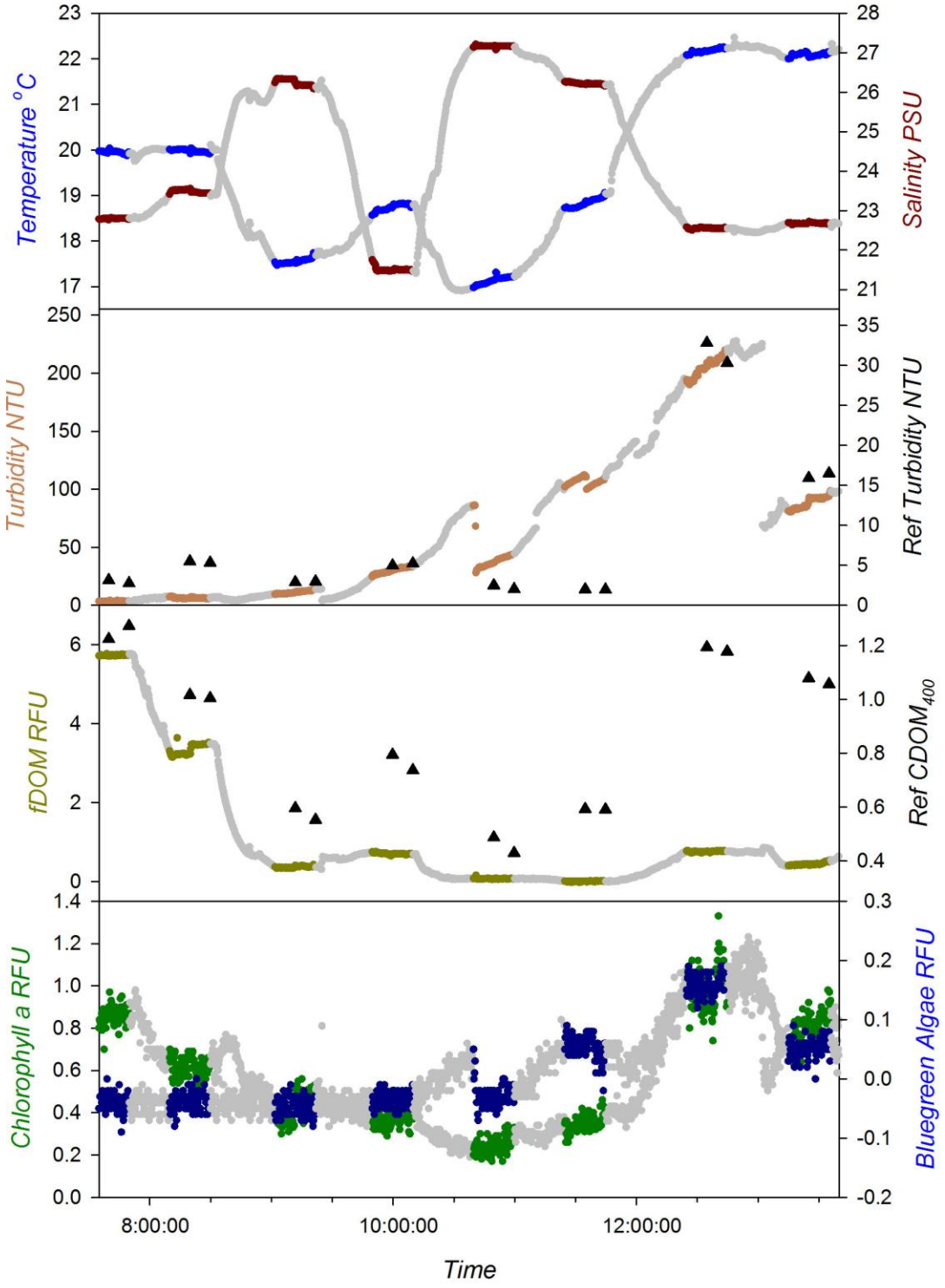



Figure 17. Time series of water conditions encountered during the surface mapping cruise in San Francisco Bay. Colored sections denote isolated sampling periods. *Top Panel:* Variation in temperature (blue) and salinity (red) at the depth of the sensors in the flow through tank, measured by an EXO 2 Sonde. *Second Panel:* Turbidity (brown) as measured by the EXO 2 and HACH 2100AN Turbidimeter analysis of reference grab samples (black triangles) taken from the exposure tank. *Third Panel:* Continuous fluorescent DOM (fDOM, olive) measured by the EXO 2, and CDOM absorbance (black triangles) measured on reference samples. *Bottom Panel:* Time series of dissolved chlorophyll a (green) and cyanobacterial (blue) fluorescence measured by the EXO 2 Sonde.

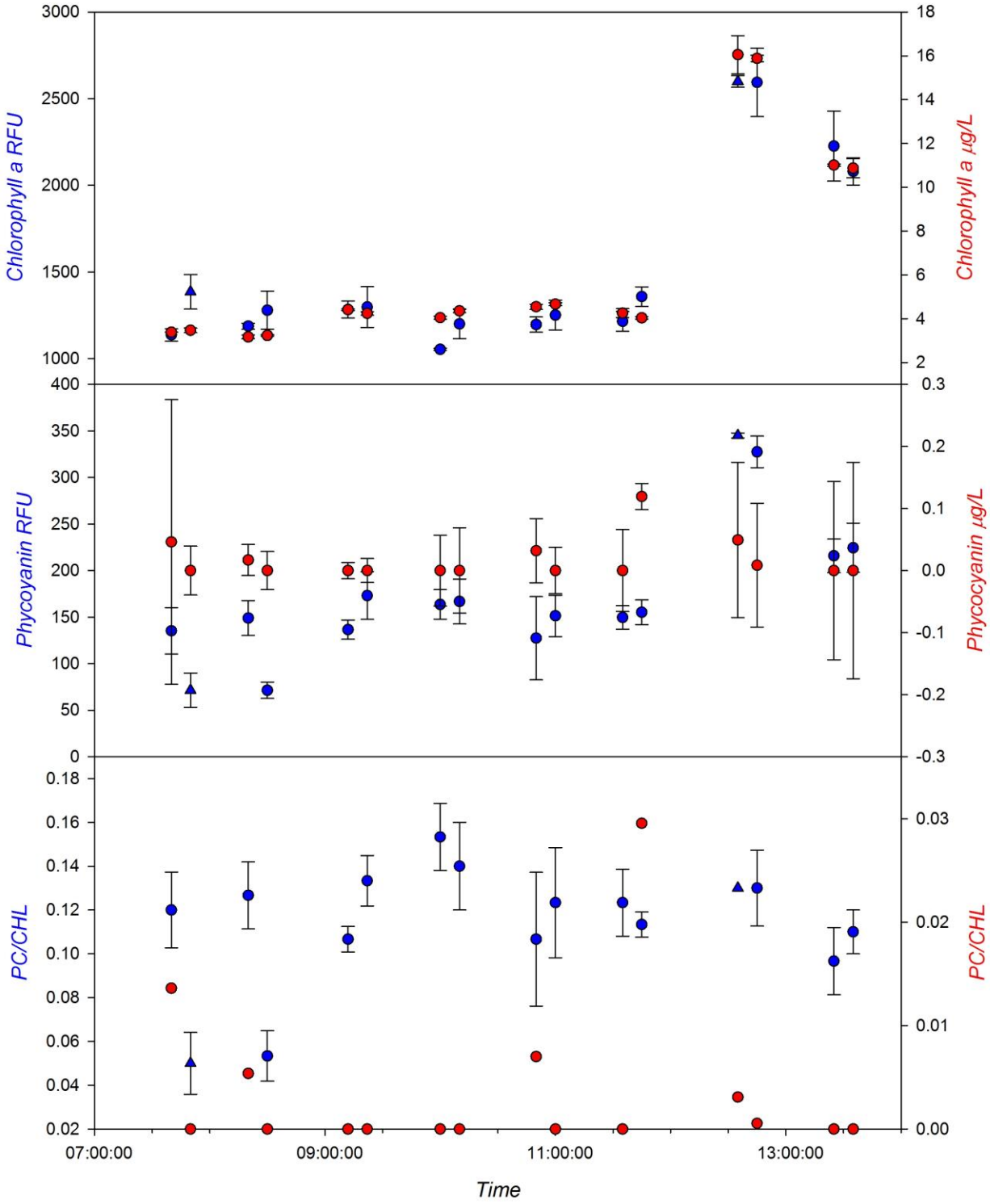



Figure 18. *Top Panel:* Plot of instrument (blue) and reference (red) measurements of chlorophyll a during San Francisco Bay surface mapping survey. Two reference measurements were made at each station, and each sample was read in triplicate on the CyanoFluor. The triangular data points represent a 2 point average due to the omission of an outlying read. *Middle Panel:* Plot of measurements of phycocyanin. *Bottom Panel:* Plot of the ratio of phycocyanin/chlorophyll a.

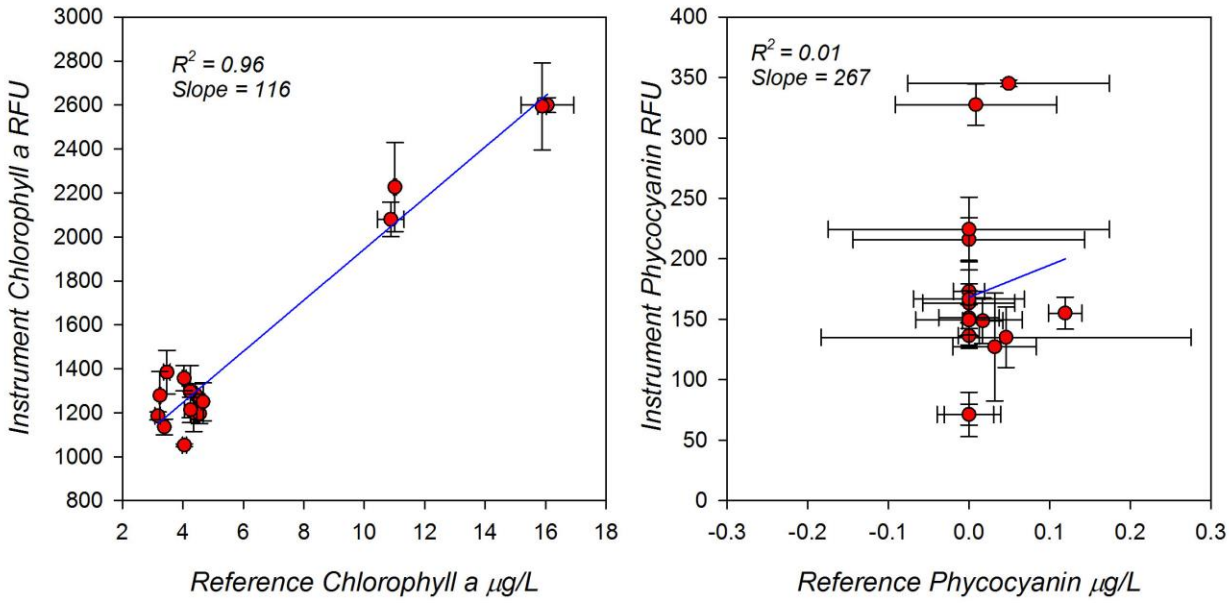



Figure 19. *Left:* San Francisco Bay surface mapping response plot of the CyanoFluor chlorophyll a measurements in RFU compared to reference chlorophyll a measured in µg/L. *Right:* Response plot of the CyanoFluor phycocyanin measurements in RFU compared to reference phycocyanin measured in µg/L.

## Monterey Bay, CA

A 75 km surface mapping cruise was undertaken in Monterey Bay, CA on 13 July 2017 using MLML's coastal research vessel the *R/V JH Martin* (*photo right*), to assess instrument performance in near-shore to oceanic water conditions. The *R/V JH Martin* was also equipped with a Headhunter StingRay continuous flow pump which drew water via a through-hull port near the bow, supplied the vessel's underway data acquisition system and was plumbed into the same exposure tank setup as described above. Flow to the tank was stopped during reference sampling, and tank water was mixed manually during the sampling process. Comparative reference samples were taken 10 and 20 min after isolation. The cruise headed out of Moss Landing Harbor, with intial samples taken near the entry to the Elkhorn Slough estuary, continuing WSW along the Monterey Bay Canyon axis to the western, oceanic edge of the bay, then NNW, back onto the shelf toward Santa Cruz, the along the 30 m isobath to assess near shore communities impacted by the combination of urban and agricultural water sheds feeding the coastal waters from Santa Cruz to Moss Landing Harbor (Fig. 20).


*R/V JH Martin*

A YSI EXO2 sonde in the tank provided continuous monitoring results during both underway and isolated time periods with measurements taken every 15 seconds (Figure 21). Continuous measurements indicated that conditions in the tank during isolation periods were a little more variable than for the previous survey. Water quality conditions along this sampling transect were in sharp contrast to the SF Bay observations and encompassed higher salinities over a narrow range (33.6 – 33.9 S) and lower temperature waters (14 – 16.5 °C). Reference samples analyses for CDOM (0.03 to 0.11) and turbidity (0.5 to 1.3 NTU) were also lower and covered a narrower range. Concentrations of extracted chlorophyll were similar in range (ca 5 – 15 µg/L) to the SF Bay observations and phycocyanin was detected at low but measurable levels (0.05 – 0.3 µg/L) at all stations indicating the presence of small marine cyanobacterial populations.

A time series of the CyanoFluor measurements of CHL, PC, and PC:CHL ratio are plotted against the corresponding reference measurements in figure 22. CHL measurements for the CyanoFluor ranged from 1635 to 3144 RFU and tracked reference sample measurements consistently. The range in PC measured by the CyanoFluor was quite small, 126 to 216 RFU, corresponding with the near detection limit for most of the reference samples. There was a little more variability among timepoint replicates for both CHL and PC in the CyanoFluor than for reference samples. Given the near detection level PC measurements, the PC:CHL ratio only ranged from 0.06 and 0.08 and values were too low to provide any meaningful comparison to reference samples.


Figure 20. Surface mapping track during the continuous underway surface mapping curise in on Monterey Bay onboard the *R/V JH Martin*. Triangles denote stations where the flow-through tank was isolated and comparative reference samples analyzed.

Cross plots of the CyanoFluor versus reference sample measurement of CHL and PC are shown in figure 23. The linear regression for CHL was significant (p=0.04) with an $R^2$=0.30 and a slope of 82 RFU/µg/L. The range of values for PC were too small to yield a significant regression (p=.24).

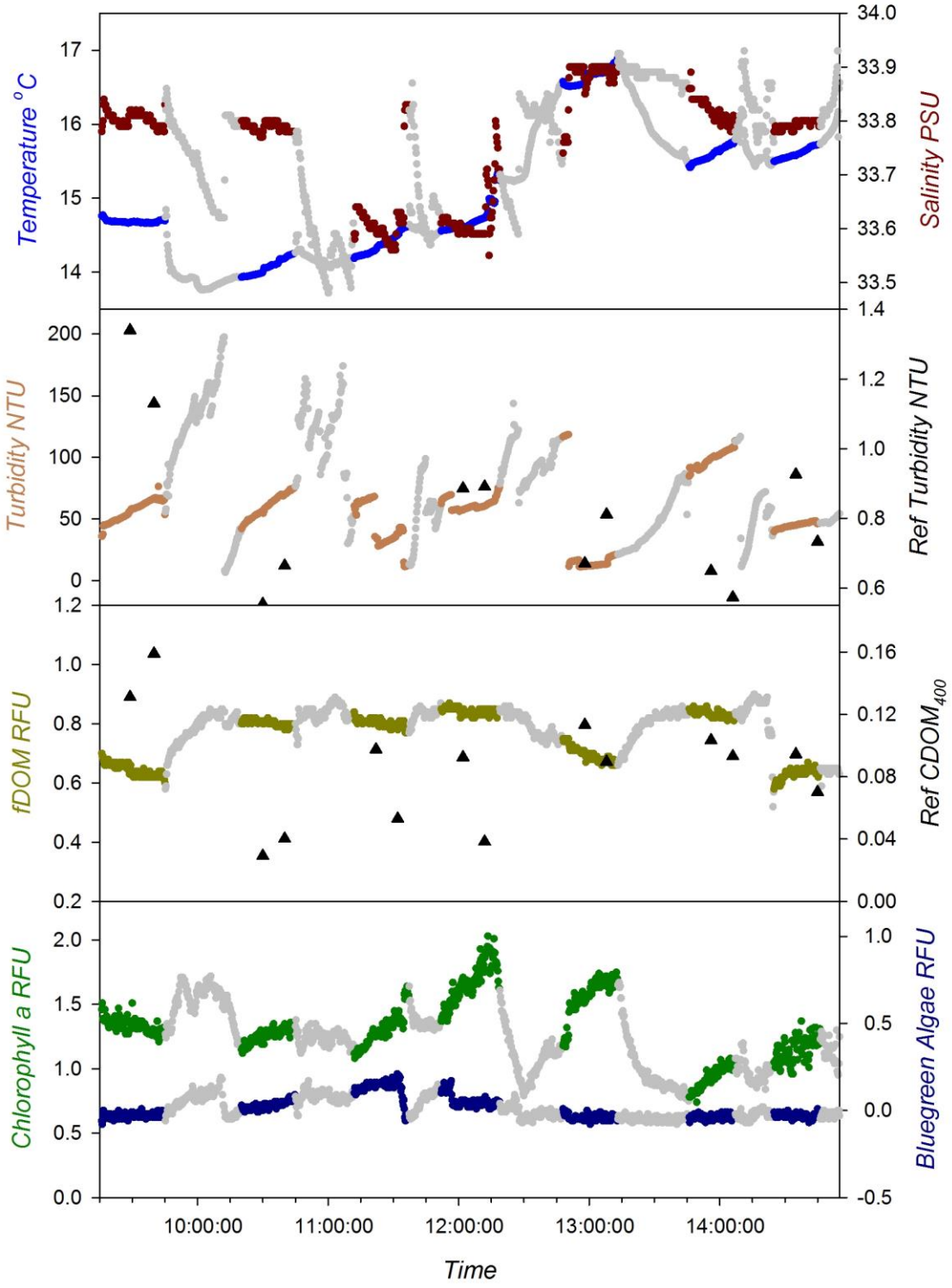



Figure 21. Water conditions encountered during the surface mapping in Monterey Bay. *Top Panel:* Variation in temperature (blue) and conductivity (red) at the depth of the sensors in the flow through tank, measured by an EXO 2 Sonde. *Second Panel:* Variation of turbidity (brown) as measured by the EXO2 and discrete samples (black triangles) taken from the tank during reference sampling and measured on a HACH 2100AN Turbidimeter. *Third Panel:* fDOM (olive) as measured by the EXO2 and CDOM measured in discrete samples on an Agilent 8453 spectrometer. *Bottom Panel:* Time series of dissolved chlorophyll a (green) and bluegreen algae (blue) as measured by the EXO2 Sonde.

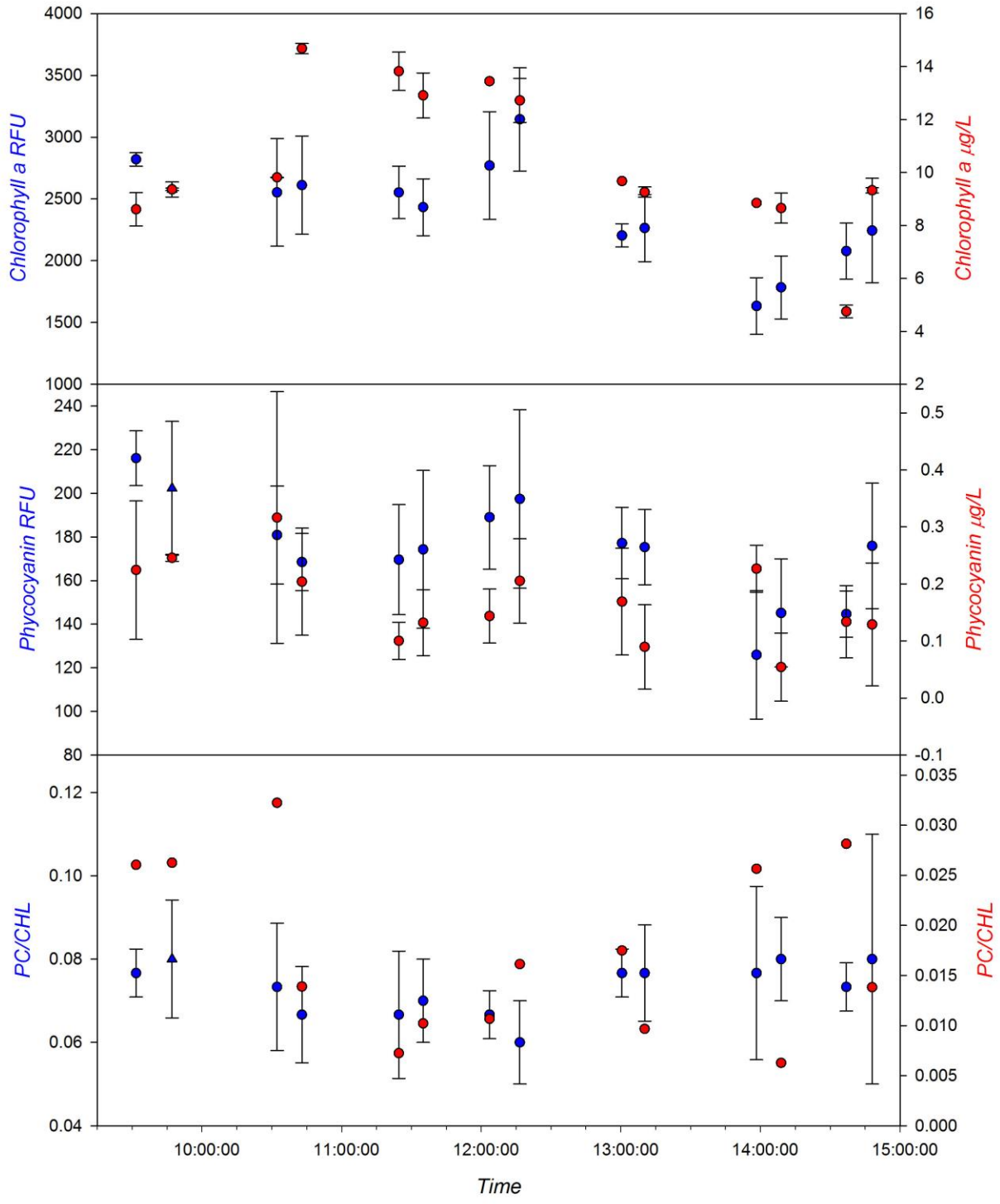



Figure 22. *Top Panel:* Plot of instrument (blue) and reference (red) measurements of chlorophyll a during Monterey Bay surface mapping. One reference measurement was made at each level while each sample was read in triplicate on the CyanoFluor, the triangles represent a 2 point average due to the omission of an outlying read. *Middle Panel:* Plot of measurements of phycocyanin. *Bottom Panel:* Plot of the ratio of phycocyanin/chlorophyll a.

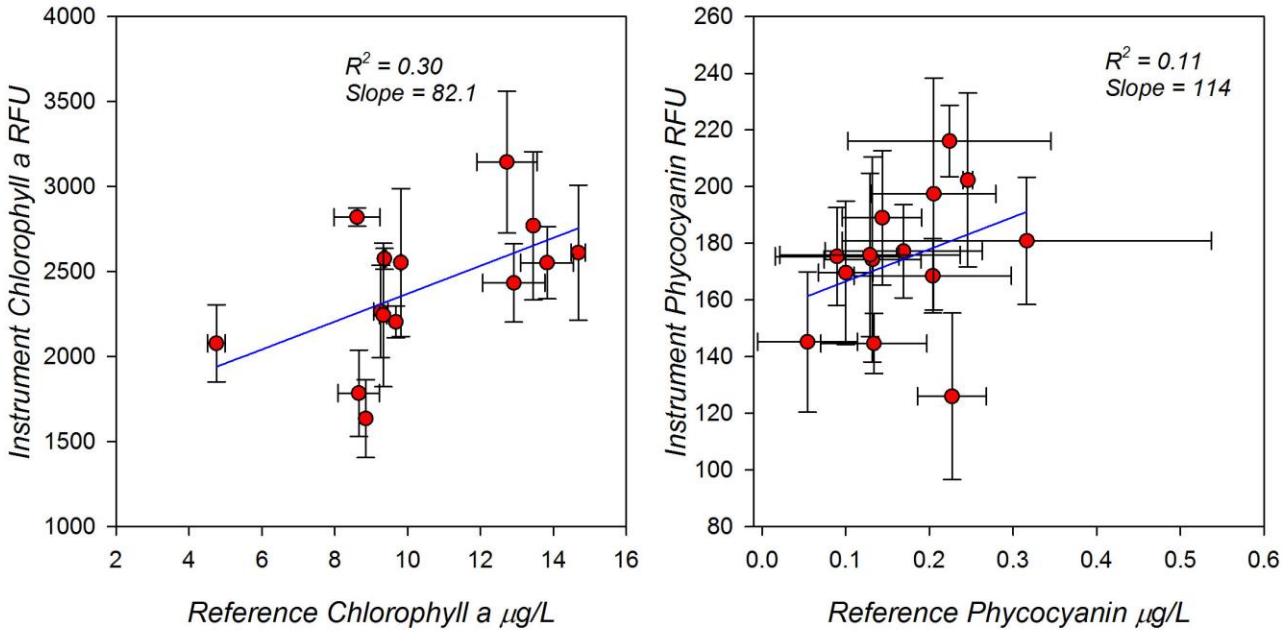
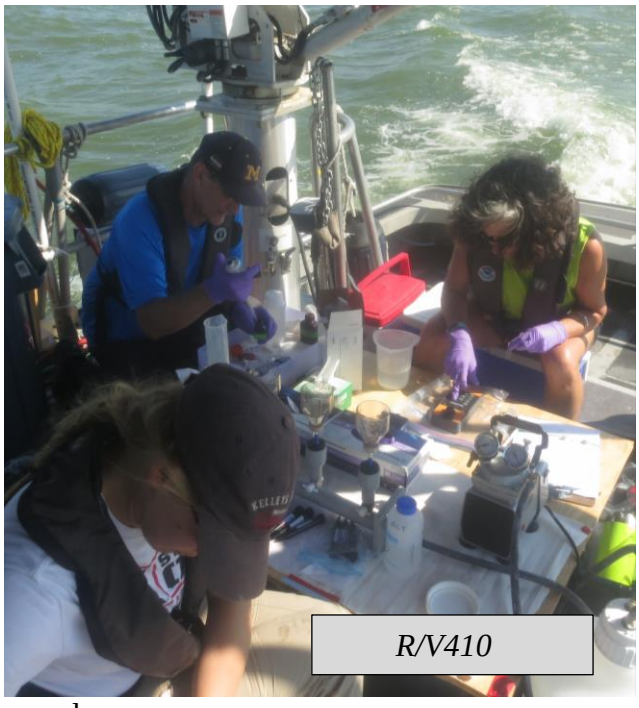



Figure 23. *Left:* Monterey Bay surface mapping response plot of the CyanoFluor chlorophyll a measurements in RFU compared to reference chlorophyll measured in µg/L. *Right:* Response plot of the CyanoFluor phycocyanin measurements in RFU compared to reference phycocyanin measured in µg/L.

## Lake Erie Surface Mapping

A surface mapping cruise was conducted in the western basin of Lake Erie on August 16th onboard the NOAA GLERL *R/V4108* (*photo at right*). The survey covered a 75 km range, including sites from the mouth of the Maumee River out to open waters 20 km offshore (Figure 24). The survey occurred during an intense cyanobacterial bloom dominated by *Microcystis*. During the underway mapping cruise seven stations were selected for comparative reference sample measurements. At each selected station, water in the tank was isolated for a period of 25 minutes, keeping it well mixed with manual stirring. After an initial equilibration period of 5 minutes, reference samples were taken at timepoints of 5 and 20 minutes from the time of isolation. An aliquot of each reference sample draw was used to expose the CyanFluor for three replicate measurements per sample.



A YSI EXO2 sonde in the tank provided continuous monitoring results during both underway and isolated time periods with measurements taken every 15 seconds (Figure 25). Continuous measurements indicated that conditions in the tank during isolation periods were more variable at high bloom stations, likely showing the colonial nature of *Microcystis* and its high buoyancy when isolated. Reference sample analyses for CDOM and turbidity are plotted over the sonde data for comparison with CDOM absorbance ranging from 0.5 to 1.7 and turbidity ranging from 2.4 to 141 NTU. During the survey, temperature ranged from 24 to 25.2 $^{o}$C and specific conductivity ranged from 260 to 370 µS/cm, reflecting a gradient in nearshore to open lake conditions. The continuous CHL and BGA sonde readings showed significant spikes in cyanobacterial abudance as the survey transited nearshore, especially outside of Maumee Bay.

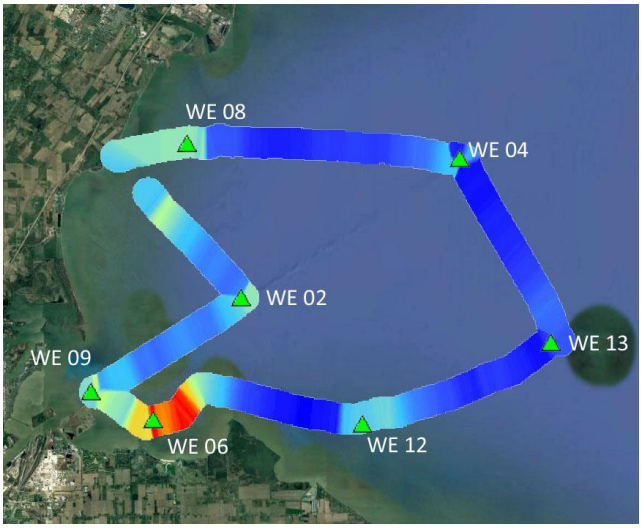



Figure 24. Surface mapping track during the surface mapping cruise in Western Lake Erie. Triangles denote stations where the flow-through tank was isolated and comparative reference samples analyzed.

A time series of the CyanoFluor measurements of CHL, PC, and PC:CHL ratio are plotted against the corresponding reference measurements in figure 26. CHL measurements for the CyanoFluor ranged from 862 to 5255 RFU and tracked reference sample measurements closely over the entire measurement range. PC concentrations measured by the CyanoFluor ranged from 237 to 35,909 RFU over the survey and again closely tracked reference samples and throughout the entire range of this severe cyanobacterial bloom. The PC:CHL ratio showed a much larger range for this study as stations included waters inside and outside of the cyanobacterial bloom. The PC:CHL measurements for the CyanoFluor ranged from 0.27 up to 8.43 at the most dense bloom site. In comparison PC:CHL ratios from the reference samples ranged from 0.07 to 0.85. The CyanoFluor appeared to be more sensitive in response in the PC channel relative to the CHL channel. This may be due to the physical arrangements of the photopigments within the cells. Given this differential response, the PC:CHL ratio will clearly not operate within an expected 0 to 1 range in terms of defining the proportion of cyanobacteria relative to total algal community.

Cross plots of the CyanoFluor versus reference sample measurements of CHL and PC are shown in figure 27. The linear regression for CHL was significant ($p=0.02$) with an $R^2=0.42$, but with a slope of only 17.9 RFU/µg/L. The much lower RFU response per unit of extracted chlorophyll was not completely unexpected since it is well known that *Microcystis* colonies like those present in this bloom have very low fluorescent responses. The linear regression for PC was highly significant ($p<0.001$) with an $R^2=0.84$ and a slope of 112 RFU/µg/L. This test was one of the first opportunities to evaluate the CyanoFluor response over true bloom conditions. A cross plot for the PC:CHL ratios was generated without including the results for station WE6, since this was such an extreme level (PC > 700 µg/L) and obscurred any meaningful relationship (Figure 28). The linear regression was significant ($p=0.02$) with $R^2 = 0.41$ and a slope of 2.92. Again the CyanoFluor response to PC was disproportianately high relative to pigment biomass.

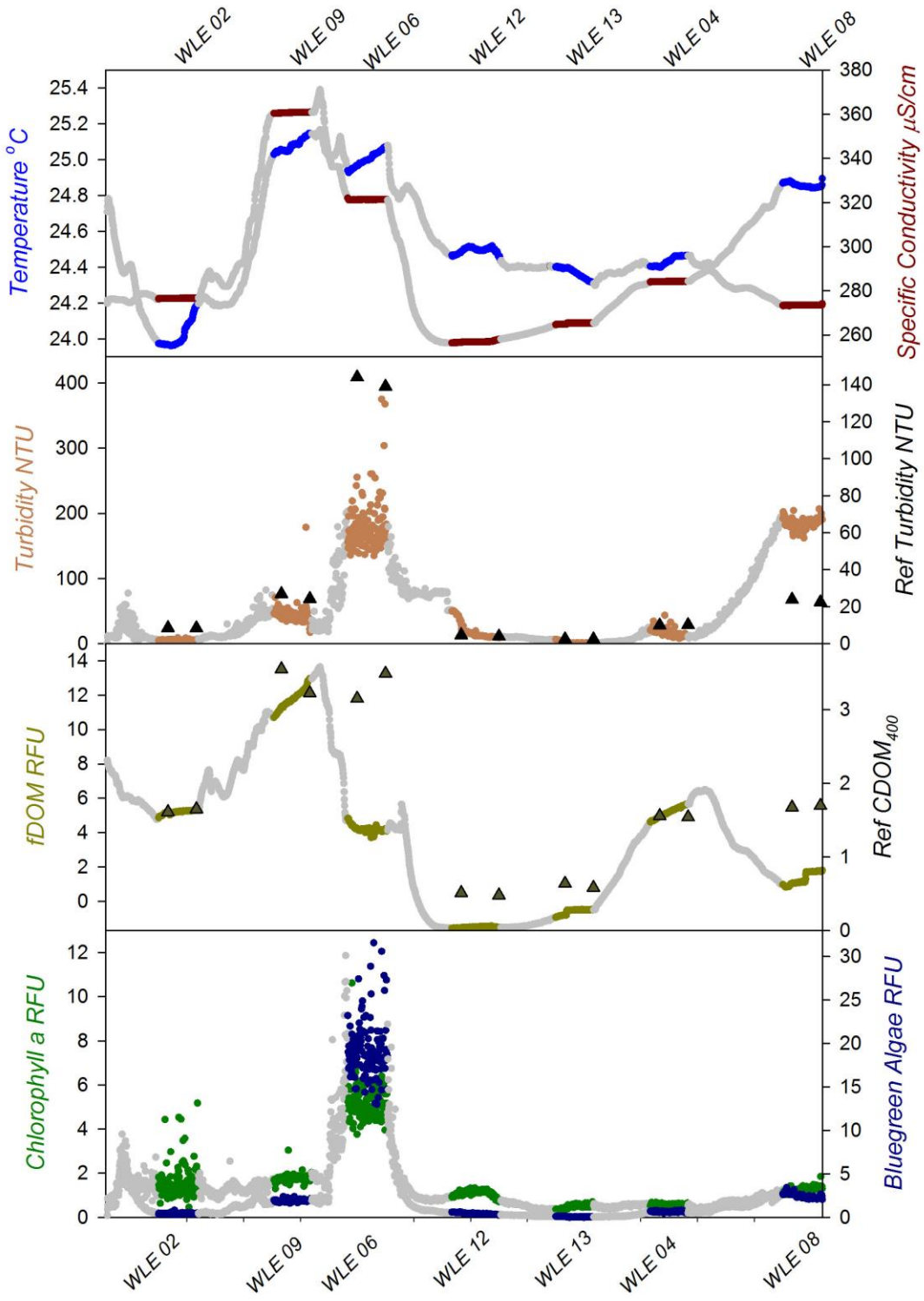



Figure 25. Water conditions encountered during the surface mapping in western Lake Erie. *Top Panel:* Variation in temperature (blue) and conductivity (red) at the depth of the sensors in the flow through tank, measured by an EXO2 Sonde. *Second Panel:* Variation of turbidity (brown) as measured by the EXO 2 and discrete samples (black triangles) taken from the tank during reference sampling and measured on a HACH 2100AN Turbidimeter. *Third Panel:* fDOM (olive) as measured by the EXO2 and CDOM measured in discrete samples on an Agilent 8453 spectrometer. *Bottom Panel:* Time series of dissolved chlorophyll a (green) and bluegreen algae (blue) as measured by the EXO2 Sonde.

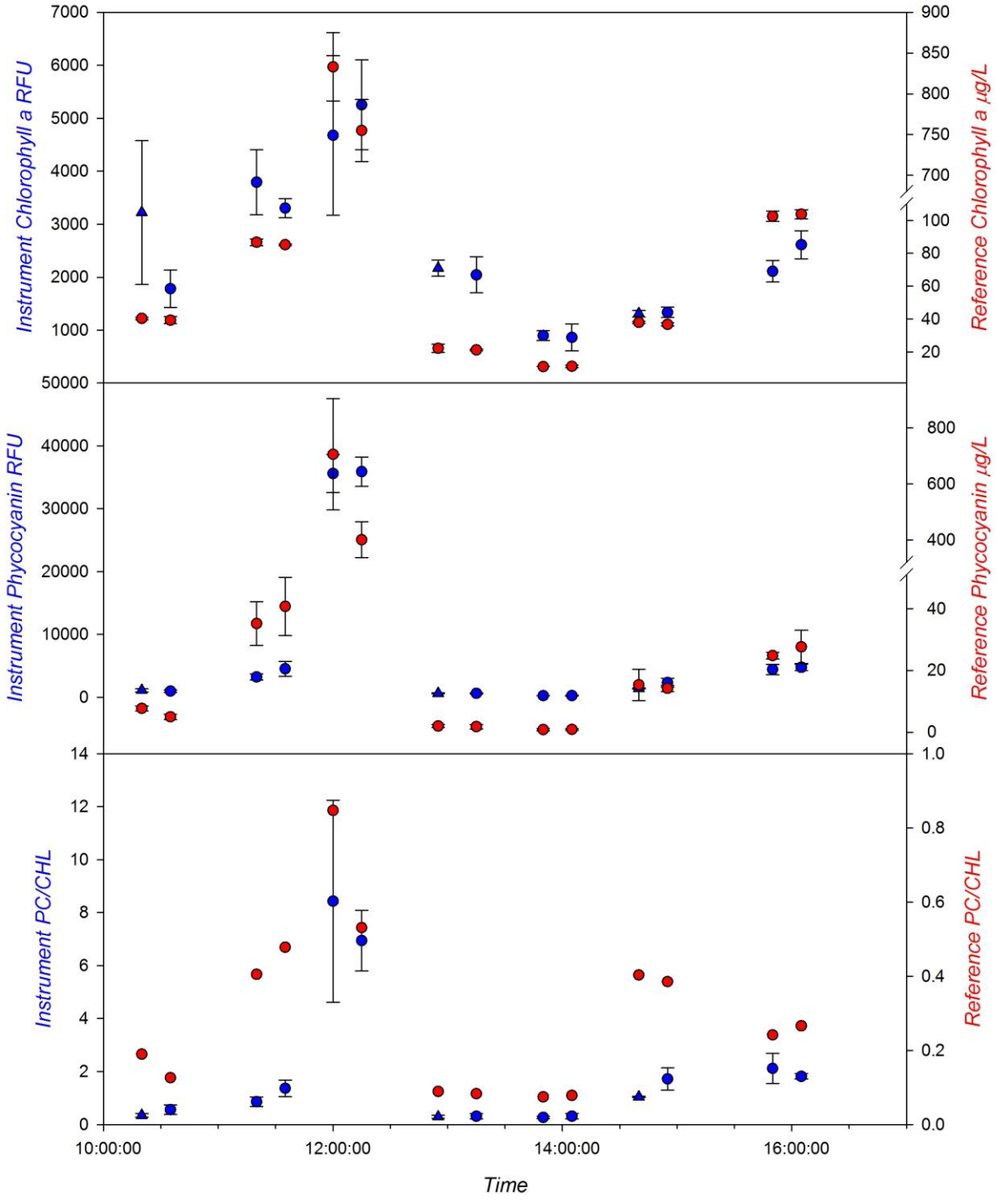



Figure 26. *Top Panel:* Time series plot of the CyanoFluor chlorophyll a in RFU (blue) and reference (red) of chlorophyll a in µg/L during surface mapping deployment on Lake Erie. Two reference measurements were made at each station, each sample was read in triplicate on the CyanoFluor, the triangles represent a 2 point average due to the omission of an outlying read. *Middle Panel:* Time series plot of the CyanoFluor phycocyanin in RFU and reference phycocyanin in µg/L *Bottom Panel:* Time series plot of the CyanoFluor phycocyanin/chlorophyll a and reference phycocyanin/chlorophyll a ratio.

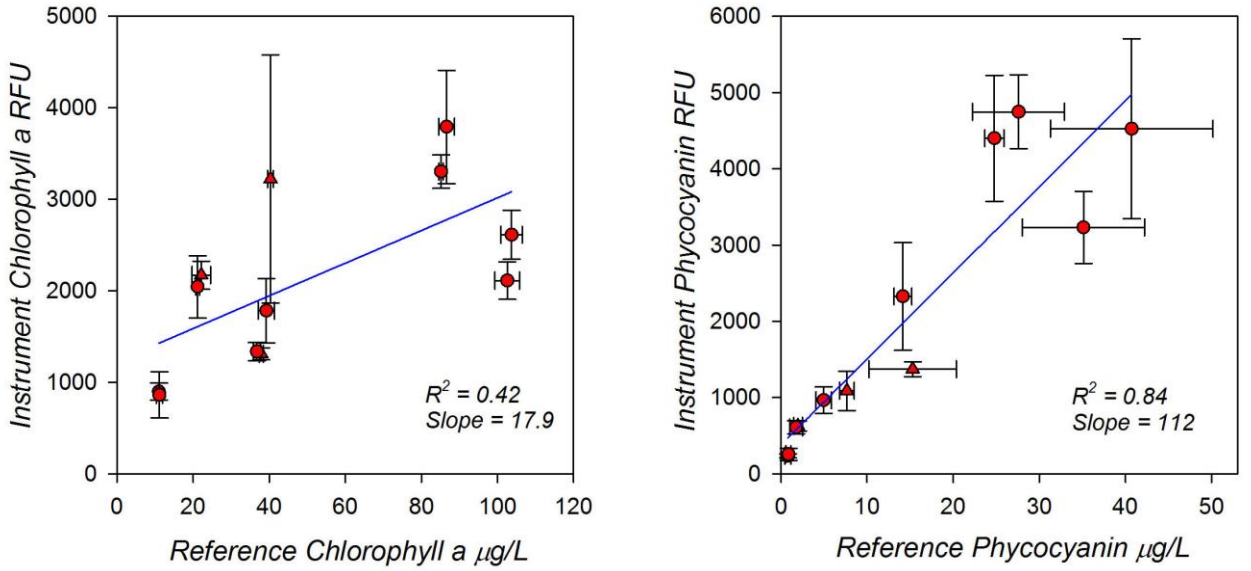



Figure 27. *Left*: Lake Erie surface mapping response plot for the CyanoFluor chlorophyll a measurements in RFU compared to reference chlorophyll a measured in µg/L. The instrument values are the average of three reads of the same sample, the triangles represent averages of two values due to the omission of outlier readings. *Right***:** Lake Erie surface mapping response plot for the CyanoFluor phycocyanin measurements in RFU compared to reference phycocyanin measured in µg/L. The instrument values are the average of three sample reads but results noted by the triangles represent an average of two values due to the omission of outlier readings.

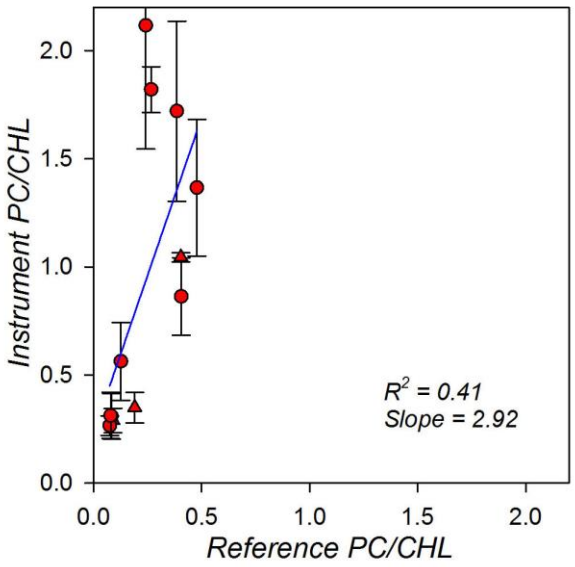



Figure 28. Lake Erie surface mapping response plot for the CyanoFluor phycocyanin/chlorophyll ratio compared to reference phycocyanin/chlorophyll ratio. The instrument values are the average of three sample reads but results noted by triangles represent an average of two values due to the omission of outlier readings.

**Field Deployment at Maumee River, Waterville, Ohio**

A 13 day field deployment in the Maumee River occurred from July 25 through August 7, at the facilities of the Bowling Green, Ohio Water Treatment Plant (Figure 29). The deployment site was located at 41.48° N, 83.74° W, in a flow-through tank located in the water treatment plant pump house. The pump house is located above the Maumee, approximately 200 m up river from the water treatment intake and approximately 35 km from the Maumee outflow into Lake Erie. River water was continuously pumped into a 180 gallon test tank where it was mixed using a shaft propeller. The residence time in the tank was approximately 10 minutes. For comparative reference samples the flow was isolated and mixed for 5 minutes prior to an instrument measurement and reference grab sample.


Figure 29. Aerial view of the Maumee River and Bowling Green Water Treatment plant (*left*) and the flow-through deployment tank servicing the supply of river water to the test instruments (*right*).

Time series results of ambient conditions for temperature, specific conductivity, turbidity, fDOM, chlorophyll and bluegreen algae measured in the flow-through tank by an EXO2 sonde are given in figure 30. Reference sample turbidity and $CDOM_{400}$ are overlaid for comparison with turbidity ranging from 21.6 to 78.3 NTU and CDOM absorbance ranging from 4.5 to 5.6. During the deployment, temperature ranged from 23.1 – 29.4ºC and discharge varied by a factor of 5x from 2000 to 10,000 cfs. The continuous sonde data indicated a 10-fold range in chlorophyll and phycocyanin RFU over the deployment, with noticeable patterns across diurnal cycles and river discharge cycles.

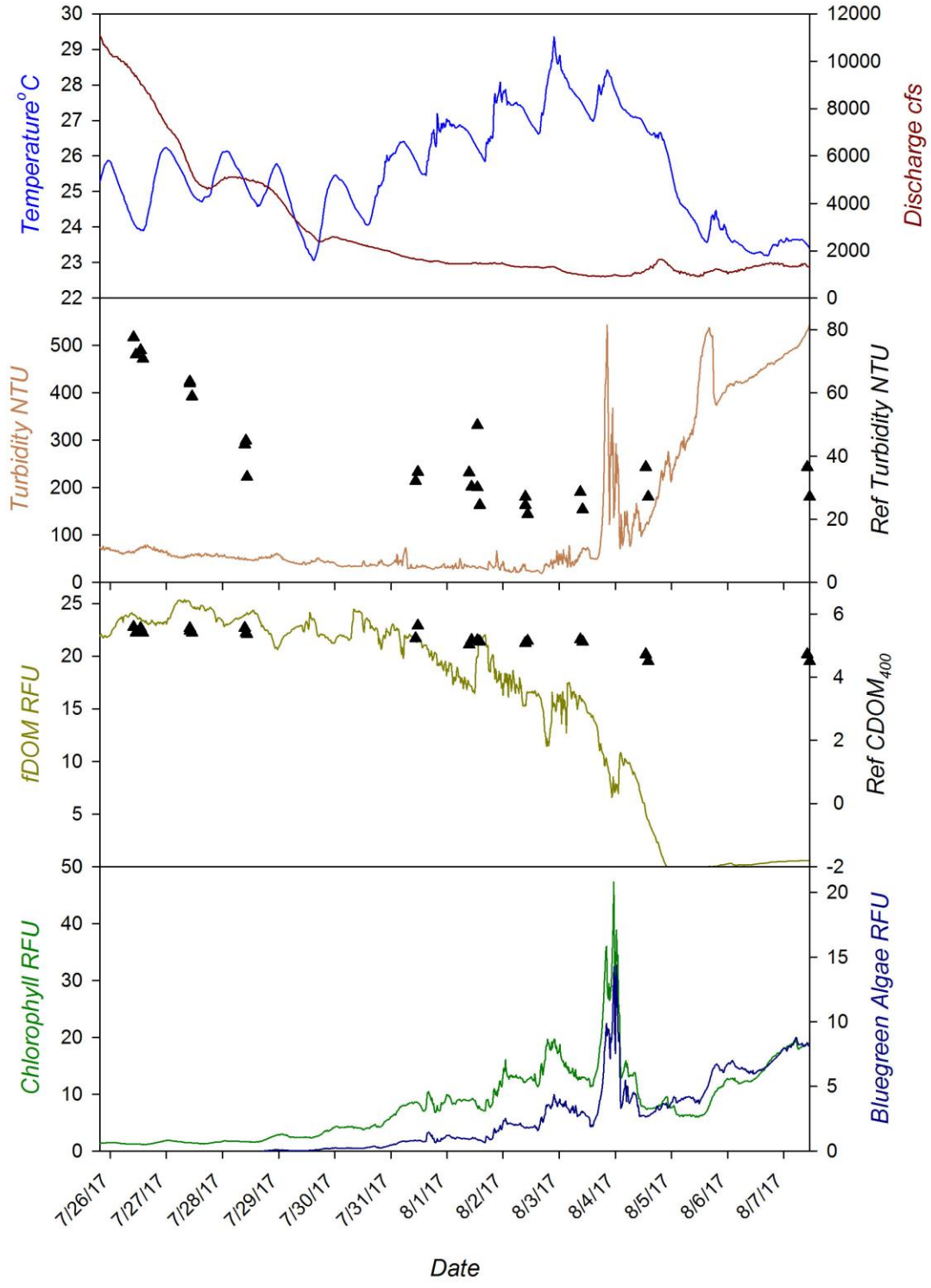



Figure 30. Environmental conditions encountered during the 13 day freshwater deployment in the Maumee River at Waterville, OH. *Top Panel:* Variation in temperature (blue) and conductivity (red) at the depth of the sensors, measured by an EXO 2 Sonde. *Second and Third Panels:* Variation of turbidity (brown) and fDOM (olive) at the depth of the sensors, measured by an EXO2 Sonde and $CDOM_{400}$ measured in discrete samples on an Agilent 8453 spectrometer (black triangles). *Bottom Panel:* Time series of chlorophyll a (green) and bluegreen algae (blue) as measured by the EXO 2 Sonde.

A time series of the CyanoFluor measurements of CHL, PC, and PC:CHL ratio are plotted against the corresponding reference measurements for the Maumee River deployment in figure 31. CHL measurements for the CyanoFluor ranged from 1374 to 14172 RFU relative to the range in reference samples of 9.5 to 119 µg/L, and in general tracked concentration patterns over the entire deployment. There were larger differences on two occassions, notably July 31 and August 1. Those dates mark a period of time when concentrations increased rapidly, but does not represent the highest levels observed. Since the agreement improved again at the following higher concentrations it would not imply any saturation effect. A nearly identical time series response was observed for the PC determiniations. PC concentrations measured by the CyanoFluor ranged from 408 to 2397 RFU, compared to a range in reference samples of 0.01 to 18.8 µg/L. Phytoplankton counts on the reference samples indicate the parallel rise in PC and CHL during that five day period was due to a sharp increase in the percentage of both Cryptophytes and Cyanobacteria. It should be noted that we also saw low but measureable levels of phycoerithythrin in the reference samples which concentrations ranging from 0 to 0.61 µg/L (not plotted). The PC:CHL ratio measured by the CyanoFluor ranged from 0.12 to 0.38 and tended to be higher than reference ratios when phytoplankton composition did not contain significant amounts of Cyanobacteria or Cryptophytes, and was lower than reference ratios when those taxa increased in abundance.

Cross plots of the CyanoFluor versus reference sample measurements of CHL and PC are shown in figure 32. The linear regression for CHL was highly significant ($p<0.001$) with an $R^2=0.92$ and a slope of 121 RFU/µg/L. The linear regression for PC was also highly significant ($p<0.001$) with an $R^2=0.86$ and a slope of 97.3 RFU/µg/L. As noted above there were two distinct clusters of values for the PC:CHL ratio's and when all the data was plotted together in a cross plot the predicted linear relationship was actually negative, which really reflects the response differences among different phytoplankton composition (Fig. 33).

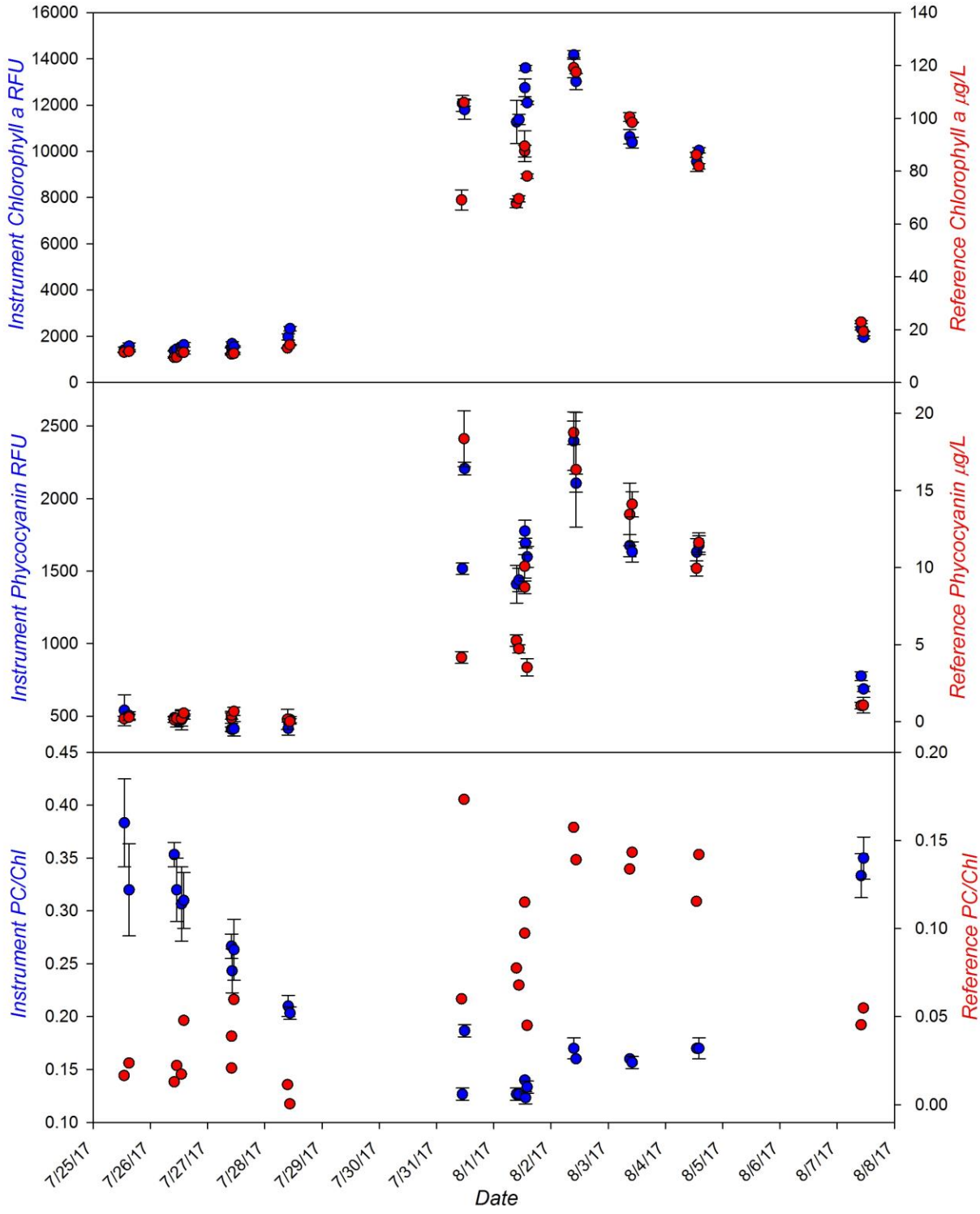



Figure 31. *Top Panel:* Time series plot of the CyanoFluor measurements in RFU (blue) and reference measurements (red) of chlorophyll a in µg/L. *Middle Panel:* Time series plot of the CyanoFluor measurements and reference measurements of phycocyanin. *Bottom Panel:* Time series plot of the CyanoFluor phycocyanin/chlorophyll a ratio and reference phycocyanin/chlorophyll a ratio during the freshwater deployment in the Maumee River at Waterville, OH. Water samples were typically collected 1 hour apart, with either two or four samples on a given day.

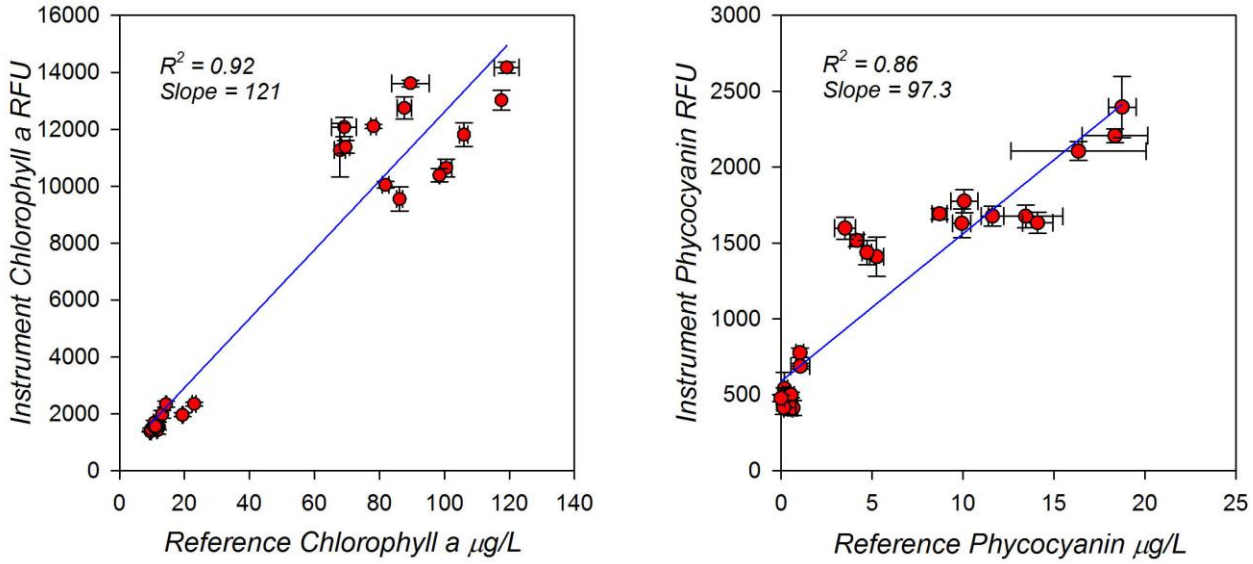



Figure 32. *Left:* Maumee River field response plot for the 13 day deployment of the CyanoFluor chlorophyll a measurements in RFU compared to reference chlorophyll a measured in µg/L. *Right:* Maumee River field response plot for the 13 day deployment of the CyanoFluor phycocyanin measurements in RFU compared to reference phycocyanin measured in µg/L.

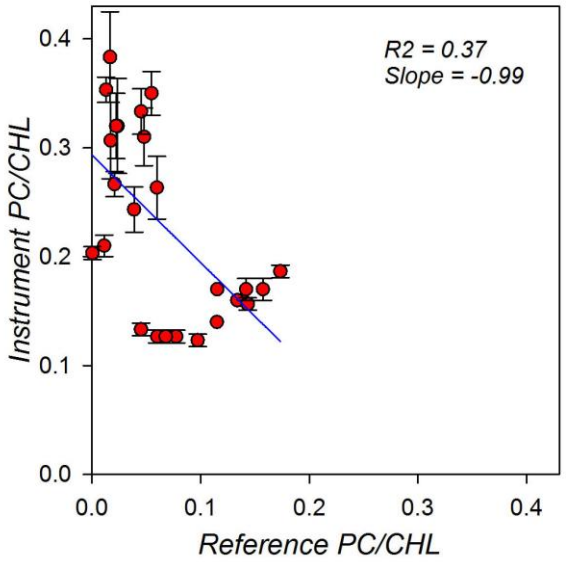



Figure 33. Maumee River field response plot for the 13 day deployment of the CyanoFluor phycocyanin/chlorophyll a ratio compared to reference sample phycocyanin/chlorophyll a ratio.

**Field Deployment at Chesapeake Biological Laboratory (CBL)**

A 28 day moored field test was conducted in Chesapeake Bay from September 6 to October 3, 2017. The deployment was located at 38.32°N, 76.45°W attached to the side of a floating pier at the mouth of the Patuxent River within Chesapeake Bay (Figure 34) The site was brackish with an average water depth of 2.2 m at the test site.

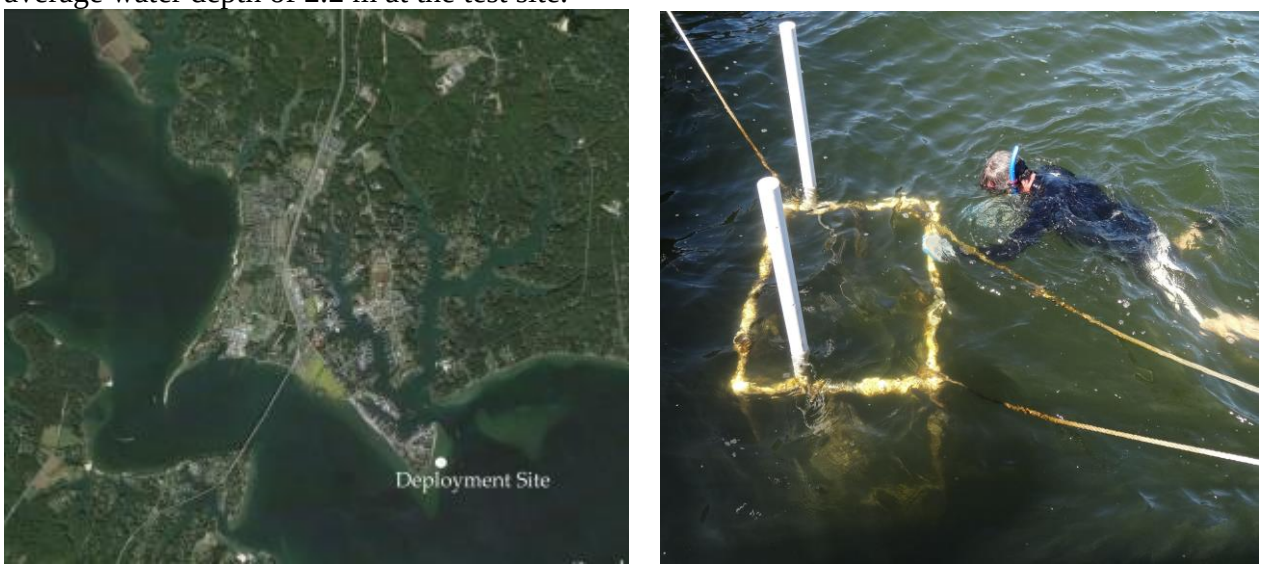



Figure 34. Aerial view of CBL deployment site (*left*) and instrument deployment rack with sensors submerged at a depth of 1 meter.

Continuous monitoring of ambient conditions for temperature, salinity, turbidity, fDOM, chlorophyll and bluegreen algae measured by an EXO2 sonde at 15 minute intervals are given in figure 35. Reference sample turbidity and $CDOM_{400}$ are overlaid for comparison with turbidity ranging from 0.7 to 2.1 NTU and CDOM absorbance ranging from 0.9 to 1.7, indicating a much less optically challenging environment than the previous river deployment. During the deployment, temperature ranged from 22.4 to 26.1°C and salinity from 8.1 to 13.2 PSU. The continuous sonde data indicated a roughly 5-fold range in chlorophyll and phycocyanin over the deployment with very strong diurnal cycles from tidal flows and a small overall decline in phycocyanin as salinity decreased.

A time series of the CyanoFluor measurements of CHL, PC, and PC:CHL ratio are plotted against the corresponding reference measurements in figure 36. CHL measurements for the CyanoFluor ranged from 820 to 9538 RFU relative to the range in reference samples of 7.4 to 21.7 µg/L, and tracked concentrations closely over the entire deployment. PC concentrations measured by the CyanoFluor showed a smaller range, 189 to 512 RFU, and generally followed the distribution of reference PC values which ranged 0.6 to 5.5 µg/L. It should be noted that this was the only site where we also saw measureable levels of phycoerithythrin in the reference samples with concentrations ranging from 0 to 3.4 µg/L (not plotted). The PC:CHL ratio measured by the CyanoFluor ranged from 0.06 to 0.21 and tended to be lower than the ratios in the reference samples which ranged from 0.06 to 0.36.

Cross plots of the CyanoFluor versus reference sample measurements of CHL and PC are shown in figure 37. The linear regression for CHL was highly significant ($p<0.001$) with an $R^2=0.78$ and a slope of 466 RFU/µg/L. The linear regression for PC was also highly significant ($p<0.001$) with an $R^2=0.55$ and a slope of 67 RFU/µg/L.

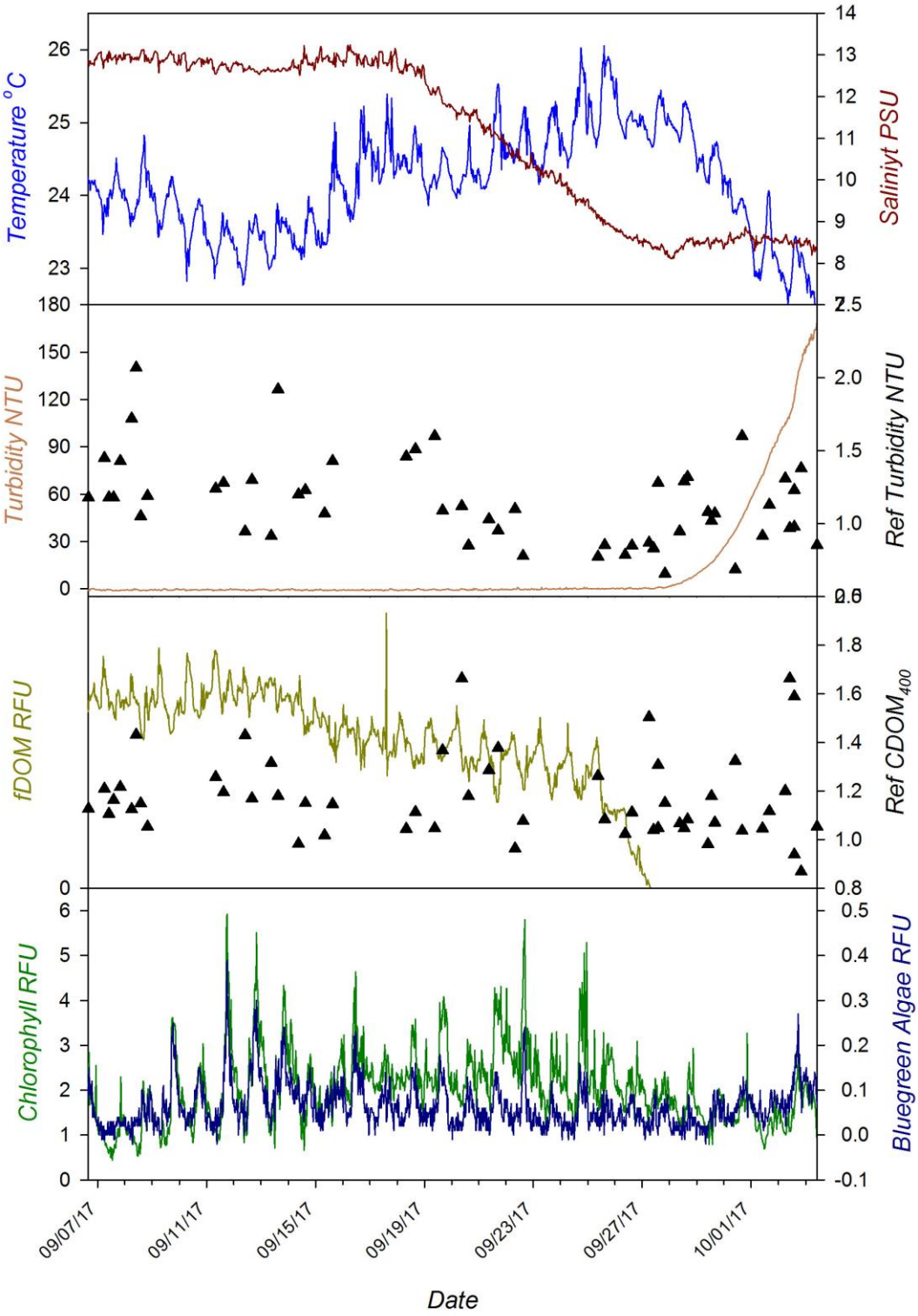



Figure 35. Environmental conditions encountered during the 28 day CBL moored deployment. *Top Panel:* Variation in temperature (green) and salinity (red) at depth of instrument sensor detected by an EXO2 sonde. *Second Panel*: Variation in turbidity (brown) as measured by the EXO2 sonde and discrete samples measured on a HACH 2100AN (black.) *Third Panel:* fDOM (dark yellow) as measured by the EXO2 and $CDOM_{400}$ measured on an Agilent 8453 spectrometer. *Bottom Panel:* Chlorophyll a (green) and bluegreen algae (blue).

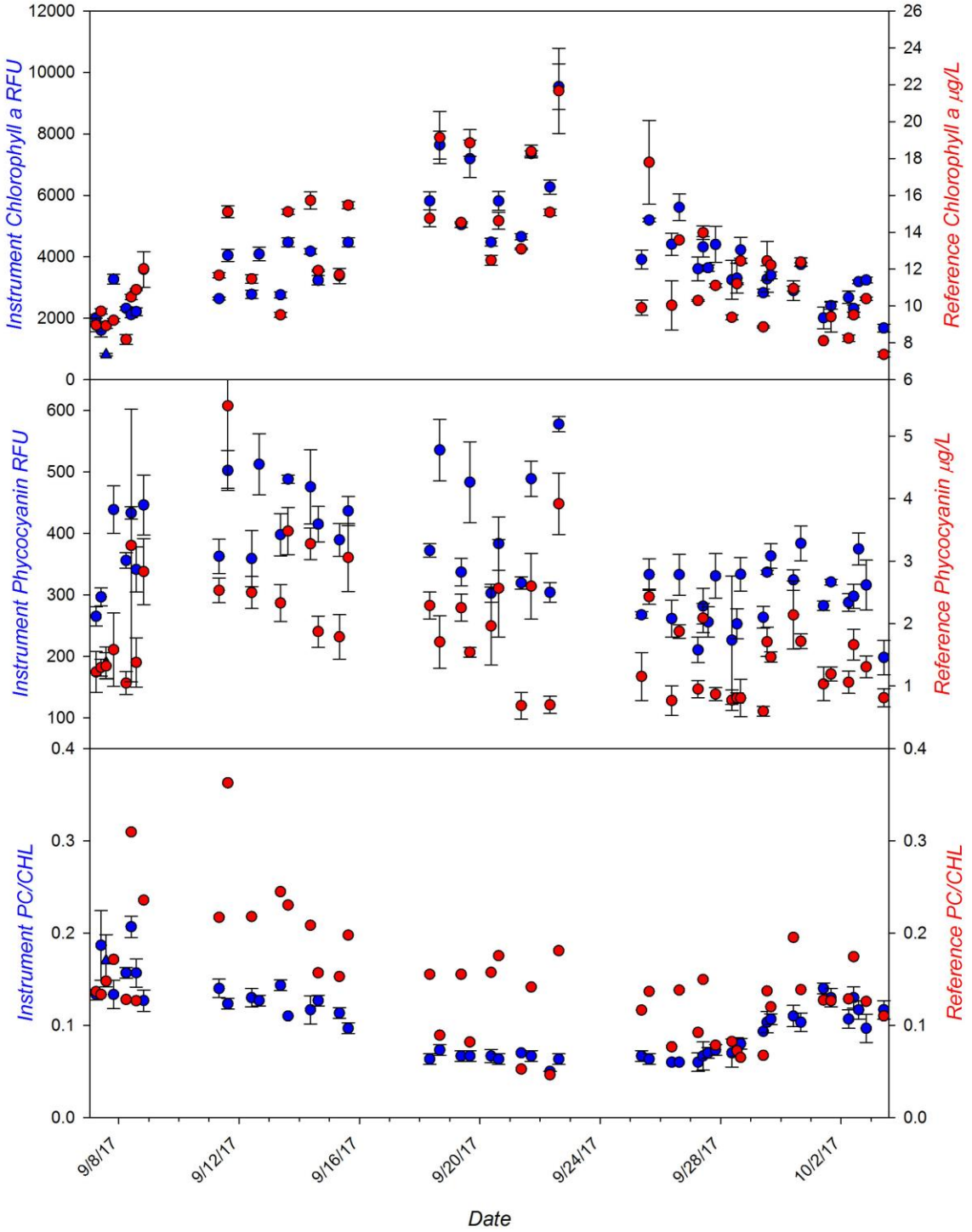



Figure 36. *Top Panel:* Time series plot of the CyanoFluor measurements in RFU (blue) and reference sample measurements (red) of chlorophyll a in µg/L *Middle Panel:* Time series plot of the CyanoFluor measurements and reference sample measurements of phycocyanin. *Bottom Panel:* Time series plot of the CyanoFluor phycocyanin/chlorophyll a ratio and reference sample phycocyanin/chlorophyll a ratio during the CBL moored deployment in Solomons, MD.

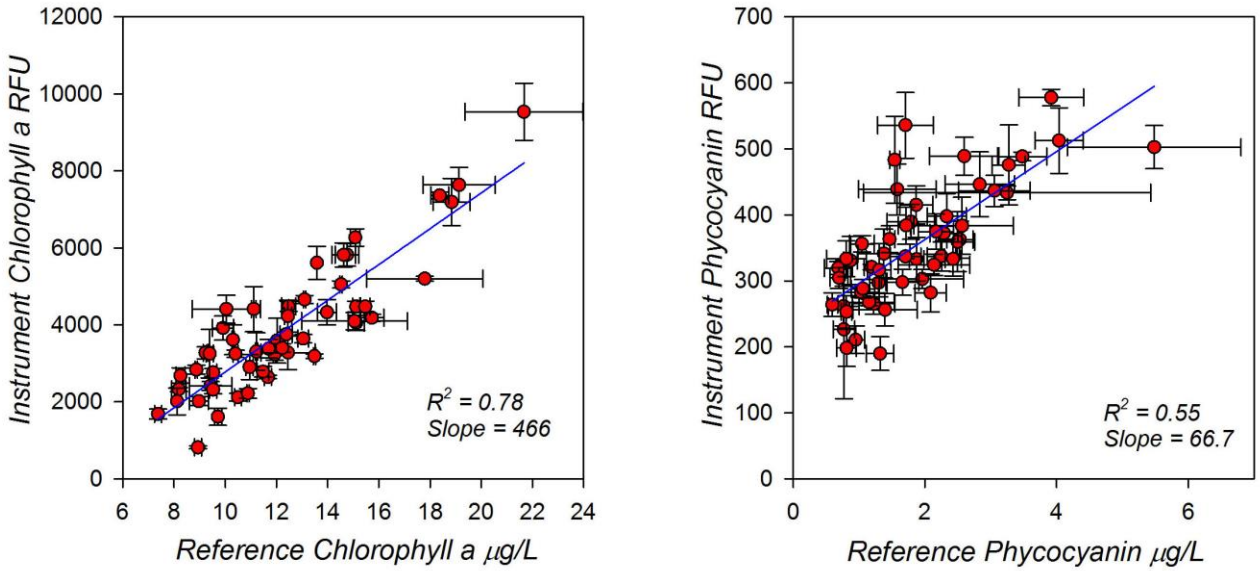
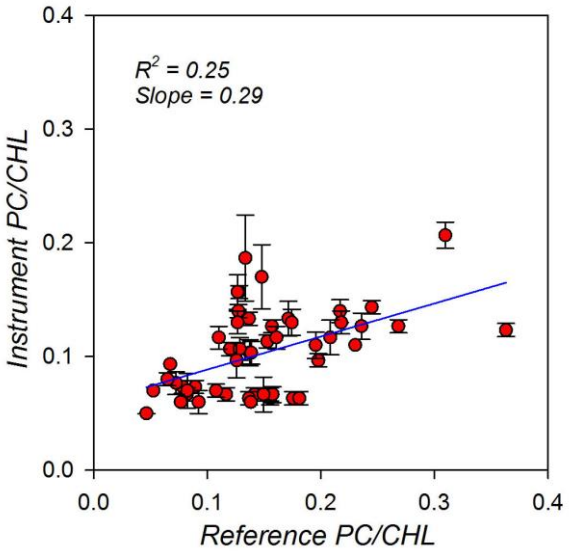



Figure 37. *Left:* CBL field response plot for the 28 day deployment of the CyanoFluor chlorophyll a measurements in RFU compared to reference chlorophyll a measured in µg/L. *Right:* CBL field response plot for the 28 day deployment of the CyanoFluor phycocyanin measurements in RFU compared to reference phycocyanin measured in µg/L.




A cross plot for the PC:CHL ratios measured by the CyanoFluor relative to the reference samples is shown in figure 38. The linear regression was highly significant ($p<0.001$) with $R^2 = 0.25$ and a slope of 0.29. In this environment the CyanoFluor response to PC was disproportianately low relative to pigment biomass.

Figure 38. CBL field response plot for the 28 day deployment of the CyanoFluor phycocyanin/chlorophyll ratio compared to reference phycocyanin/chlorophyll.

## GLOBAL RESPONSE

One-to-one cross plots of CyanoFluor versus reference sample determinations for all field tests were combined into a single set of plots for CHL, PC, and PC:CHL ratios (Fig. 39). Data from each field test are color coded so that the variance in fluorescence response across different environments and phytoplankton communites can be observed. The variation in response for CHL was quite large across environments with response slopes varying from a minimum of 17.9 RFU/µg/L for Lake Erie to 466 RFU/µg/L for Chesapeake Bay (Table 8). The variance in response slopes for PC was much less (Table 8), likely because most of the spread in biomass occurred within the Great Lakes studies where PC was dominated by a common taxa. For these data a single regression line could be gerenated ($p<0.001$) with an $R^2=0.88$ and a slope of 118 RFU/µg/L (Fig. 39).

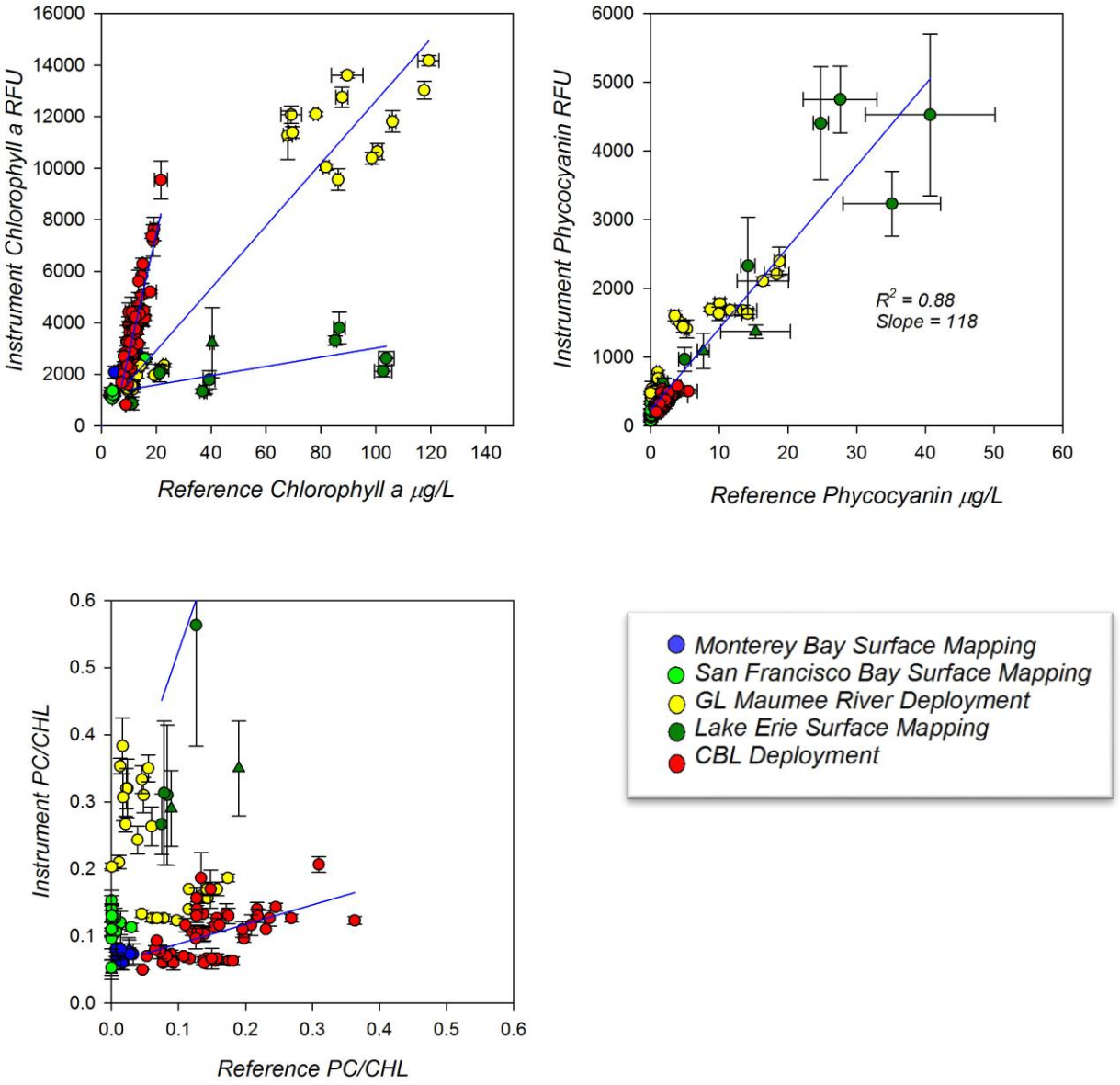



Figure 39. Global response plot for the CyanoFluor PC/CHL observed during the five ACT field trials. The blue lines represent distinct linear response regressions if present.

Given the difference in CHL versus PC responses, the PC:CHL ratios were very different across environments with slopes of 0.29 and 2.9 for CBL and Lake Erie, respectively (Fig. 39 and Table 8).

Table 8. Summary of CHL and PC response curves of the CyanoFluor calculated as the slope of CyanoFluor readings regressed against extracted pigment concentrations from comparative reference samples. (ns, denotes regression was not significant)

| Test | CHL Response (RFU/µg/L) | PC Response (RFU/µg/L) | PC:CHL |
|---|---|---|---|
| MLML SW Lab 6/27 | 156 | 308 | ns |
| MLML FW Lab 6/28 | 417 | 85 | ns |
| MLML FW Lab 6/29 | 313 | 108 | ns |
| Great Lake FW Lab | 134 | 32 | ns |
| SF Bay mapping | 116 | 267 | ns |
| MB mapping | 82 | 114 | ns |
| WLE mapping | 18 | 112 | 2.92 |
| Maumee River mooring | 121 | 97 | -0.99 |
| CBL mooring | 466 | 67 | 0.29 |

**Quality Assurance and Quality Control**

All technology evaluations conducted by ACT comply with its Quality Management System (QMS), which includes the policies, objectives, procedures, authority, and accountability needed to ensure quality in work processes, products, and services. A QMS provides the framework for quality assurance (QA) functions, which cover planning, implementation, and review of data collection activities and the use of data in decision-making, and quality control. The QMS also ensures that all data collection and processing activities are carried out in a consistent manner, to produce data of known and documented quality that can be used with a high degree of certainty by the intended user to support specific decisions or actions regarding technology performance. ACT's QMS meets U.S. Environmental Protection Agency quality standards for environmental data collection, production, and use, and the requirements of ISO/IEC 17025:2005(E), *General Requirements for the Ccompetence of Testing and Calibration Laboratories*.

Quality Control Sample Analysis

As part of the sample analysis quality control evaluation two field blank samples (Table 9) and two field duplicate samples (Tables 10) were collected during each of the moored field testing applications in the Maumee River and in Chesapeake Bay. Results of the reference sample field blanks (Table 9) were quite consistent across all samples at both sites and did not indicate the presence of any contamination or bias associated with sample processing or analysis.

Table 9. Results of reference sample Field Blank analysis.

| Sample ID | Collection | Turbidity (NTU) | CDOM ($A_{400}$) | CHLa (µg/L) (stdev) | PC (µg/L) (stdev) | PE (µg/L) (stdev) |
|---|---|---|---|---|---|---|
| GL14 | 7/28/17 10:00 | 0.097 | 0.02 | 0.02 (0.02) | 0.20 (0.22) | -0.23 (.002) |
| GL24 | 8/2/17 09:30 | 0.08 | 0.08 | 0.07 (0.03) | -0.06 (0.08) | -0.28 (.002) |
| | | | | | | |
| CBL39 | 9/27/17 10:00 | 0.23 | 0.07 | 0.04 (0.01) | -0.17 (0.15) | -0.14 (0.002) |
| CBL55 | 10/2/17 10:00 | 0.18 | 0.06 | 0.03 (0.01) | -0.17 (0.15) | -0.06 ((0.08) |

Results of the laboratory analysis for reference sample field duplicates (Table 10) were quite consistent across all samples at both sites and did not indicate the presence of any contamination or bias associated with sample processing or analysis. Coefficients of variance were elevated when concentrations were low or near detection limits.

**Table 10**. Comparison of reference sample analysis for Field Duplicates from the Maumee River, OH and Chesapeake Biological Laboratory, MD mooring tests.

| Sample ID | Sample Type | Turbidity (NTU) | CDOM ($A_{400}$) | CHLa (µg/L) | PC (µg/L) | PE (µg/L) |
|---|---|---|---|---|---|---|
| GL10 | Ref | 63.6 | 5.46 | 10.9 | 0.23 | 0.036 |
| GL11 | Field Dup | 63.0 | 5.54 | 10.7 | 0.42 | 0.046 |
| | **Mean (stdev)** | **63.3 (0.42)** | **5.51 (0.06)** | **10.8 (0.10)** | **0.32 (0.13)** | **0.04 (0.01)** |
| | **Coeff Var** | **0.67** | **1.08** | **0.94** | **41.9** | **16.9** |
| | | | | | | |
| GL20 | Ref | 30.3 | 5.17 | 87.6 | 10.1 | 0.14 |
| GL21 | Field Dup | 30.1 | 5.15 | 89.5 | 8.7 | 0.11 |
| | **Mean (stdev)** | **30.2 (0.14)** | **5.16 (0.01)** | **88.6 (1.3)** | **9.4 (1.0)** | **0.12 (0.03)** |
| | **Coeff Var** | **0.47** | **0.20** | **1.5** | **10.3** | **20.7** |
| | | | | | | |
| CBL41 | Ref | 1.28 | 1.31 | 12.7 | 1.62 | 1.39 |
| CBL42 | Field Dup | 1.28 | 1.05 | 13.4 | 1.18 | 0.96 |
| | **Mean (stdev)** | **1.28 (0.00)** | **1.18 (0.18)** | **13.1 (0.44)** | **1.40 (0.31)** | **1.18 (0.3)** |
| | **Coeff Var** | **0.00** | **15.6** | **3.4** | **22.4** | **25.5** |
| | | | | | | |
| CBL57 | Ref | 0.98 | 1.59 | 13.4 | 2.01 | 3.25 |
| CBL58 | Field Dup | 1.23 | 0.94 | 13.6 | 2.33 | 2.75 |
| | **Mean (stdev)** | **1.10 (0.18)** | **1.26 (0.46)** | **13.5 (0.18)** | **2.17 (0.23)** | **3.00 (0.35)** |
| | **Coeff Var** | **16.1** | **36.4** | **1.4** | **10.6** | **11.8** |

Technical System Audits

A TSA is a thorough, systematic, on-site qualitative audit of sampling and measurement processes and procedures associated with a specific technology evaluation. The objectives of the TSAs conducted during this evaluation were to assess and document the conformance of on-site testing procedures with the requirements of the Test Protocols, the ACT Quality Assurance Project Plan (QAPP), and associated Standard Operating Procedures (SOPs).

The TSA's were conducted in accordance with the procedures described in the EPA's *Guidance on Technical Audits and Related Assessments for Environmental Data Operations (EPA QA/G-7)* and ISO 19011, *Guidelines for Quality and/or Environmental Management Systems Auditing*. A TSA checklist based on the Test Protocols was prepared prior to the audits and reviewed by the ACT Director and Senior Scientist. The TSA assessed ACT personnel, the test and analytical facilities, equipment maintenance and calibration procedures, sample collection, analytical activities, record keeping, and QC procedures. Reference sample handling and chain-of-custody were observed during each audit.

During the audits, the QA Manager met with ACT technical staff involved in the evaluation and asked them to describe the following procedures. All procedures were observed and logbooks, data forms, and other records were reviewed.

Key components of the audit included:

- Assessment of Quality Assurance/Quality Control:
  - Adequacy of procedures, and
  - Adherence to procedures.
- Assessment of Sample System:
  - Sample collection,
  - Analytical procedures, and
  - Documentation.
- Assessment of Data and Document Control:
  - Chain of custody, and
  - Documentation.

The TSA's findings were positive for the two field tests, which were implemented consistent with the Test Protocols, QAPP, and SOPs. Minor deviations were documented in laboratory records. There were no deviations that may have had an effect on data quality for these tests.

Prior to the start of the laboratory test, the stock cultures of individual species of marine algae were contaminated. The algal cultures were grown in a semi-continuous culture system. The culture vessels were filled with filtered seawater pumped in through the MLML seawater system. The pore size of the filters allowed other species of algae to enter the cultures. Corrective action, replacing the existing filters with filters with a smaller pore size, was taken immediately and effectively resolved the problem. This resulted in a number of deviations in the Test Protocols. The deviations and corrective action altered the type of data results but did not have an effect on data quality.

For all tests, the implementation of the audited tests was performed in a manner consistent with ACT data quality goals. All samples were collected as described in the Test Protocols and SOPs. Examination of maintenance and calibration logs provided evidence of recent and suitable calibration of sampling and analytical equipment. The overall quality assurance objectives of the tests were met.

ACT personnel are well-qualified to implement the evaluation and demonstrated expertise in pertinent procedures. Communication and coordination among all personnel was frequent and effective. Internal record keeping and document control was well organized. The ACT staff understands the need for QC, as shown in the conscientious development and implementation of a variety of QC procedures.

Data Quality Review

Quality Control. Quality control samples collected included periodic duplicate field samples and field blanks to determine the adequacy and control of field collection and processing procedures of analytical laboratory processing and analysis procedures. QC samples were treated identically to routine samples in terms of sample identification, custody, request for analytical services, and data processing.

Results from field blanks showed no contamination, indicating that field procedures were adequate for accomplishing data quality objectives. If the concentration observed in a replicate did not meet the criteria for precision and accuracy, the value was rejected and a back-up filter was processed and analyzed.

Calibration data were reviewed at a cursory level and was determined to be acceptable. No data qualification was required based on the calibration review.

Custody for all reference samples was adequately maintained throughout the collection, processing, and delivery of samples to the analytical laboratories. Chain-of-custody documentation was complete. All analysis holding times were met as described in SOPs for the method or the Test Protocols.

Overall, data quality for the reference water samples was acceptable.

Data Verification, Validation, and Quality Assessment. Data review is conducted to ensure that only sound data that are of known and documented quality and meet technology evaluation quality objectives are used in making decisions about technology performance. Data review processes are based in part on two EPA guidance documents: *Guidance on Environmental Data Verification and Data Validation* (QA/G-8) [EPA, 2002] and *Guidance on Technical Audits and Related Assessments for Environmental Data Operations* (QA/G-7) [EPA, 2000].

The data were verified and validated to evaluate whether the data have been generated according to the Test Protocols and satisfied acceptance criteria. Data verification evaluates the completeness, correctness, and consistency of the data sets against the requirements specified in the Test Protocols, measurement quality objectives (MQOs), and any other analytical process requirements contained in SOPs. Data validation assesses and documents compliance with methods and procedures and determines the quality of the data based on the quality objectives defined in the Test Protocols and QAPP.

The ACT QA Manager reviewed the reference data sets from all field and laboratory tests. The number of reference samples collected at each site and the laboratory tests are in Table 12. 243 reference samples were collected for the field and laboratory tests. Each reference sample was split into replicates for pigment analysis. Distinct grab samples were taken for phytoplankton cell counts, CDOM, and turbidity.

Table 12. Number of samples and replicates collected and the pigment analysis for each field and lab test.

| Site | No. of Samples[1/] | No. of Replicates per Sample[2/] | No. of Measurements (Pigments)[3/] |
|---|---|---|---|
| MLML – Lab tests | 98 | 5 | 490 |
| SF Bay – Surface mapping | 16 | 5 | 80 |
| Monterey Bay – Surface | 14 | 5 | 70 |
| Maumee River Moored test | 31 | 5 | 155 |
| Lake Erie - Surface | 14 | 5 | 70 |
| Great Lakes – Lab test | 10 | 5 | 50 |
| CBL – Moored test | 60 | 5 | 300 |
| Total | 243 | | 1,215 |

1/ Includes replicate samples
2/ A total of six replicates were filtered for each reference sample. Pigment analysis was conducted on two replicates chlorophyll and three replicates for phycobilins. One filter was reserved in storage.
3/ Does not include phytoplankton cell counts and biovolume, CDOM, and turbidity, which also were verified and validated.

The data verification determined that the sampling and analysis protocols specified in the Test Protocols were followed, and that the ACT measurement and analytical systems performed in accordance with approved methods, based on:

- The raw data records were complete, understandable, well-labeled, and traceable;
- All data identified in the Test Protocols were collected;
- QC criteria were achieved; and
- Data calculations were accurate.

Data validation uses the outputs from data verification and included inspection of the verified field and laboratory data to determine the analytical quality of the data set. Validation of the data sets established:

- Required sampling methods were used;
- Sampling procedures and field measurements met performance criteria; and
- Required analytical methods were used.

The data validation also confirmed that the data were accumulated, transferred, summarized, and reported correctly. There is sufficient documentation of all procedures used in the data collection and analysis to validate that the data were collected in accordance with the evaluation's quality objectives.

Data Quality Assessment, sometimes referred to as a Data Usability Assessment, is a scientific and statistical evaluation of validated data to determine if the data are of the right type, quality, and quantity to support conclusions on the performance of the technologies. The DQA determined that the test's data quality objectives, described in Section 7.2 of the Test Protocols and Section 3.4 and Appendix B of the ACT QAPP (ACT, 2016), were achieved. This evidence supports conclusions

that:

- The sampling design performed very well and is very robust with respect to changing conditions.
- Sufficient samples were taken to enable the reviewer to see an effect if it were present.
- The complete data set was fit for its intended use for determining the performance of the test instruments.

Audit of Data Quality. The ACT QA Manager also conducted an Audit of Data Quality (ADQ) on verified data to document the capability of ACT's data management system to collect, analyze, interpret, and report data as specified in the Test Protocols, QAPP, and SOPs. An ADQ involves tracing data through their processing steps and duplicating intermediate calculations. A representative set of approximately 10% of the data was traced in detail from 1) raw data from field and laboratory logs, 2) data transcription, 3) data reduction and calculations, to 4) final reported data.

The ADQ determined that the data were accumulated, transferred, reduced, calculated, summarized, and reported correctly. There is sufficient documentation of all procedures used in the data collection and analysis to verify that the data have been collected in accordance with ACT quality objectives defined in the ACT QMS.

## REFERENCES

Speziale, B.J., Schreiner, S.P., Giammatteo, P.A., and Schindler, J.E. 1984. Comparison of *N,N*-Dimethylformamide, Dimethl Sulfoxide, and Acetone for Extraction of Phytoplankton Chlorophyll. Canadian Journal of Fisheries and Aquatic Sciences, 41:1519-1522.

Lawrenz, E., Fedewa, E.J. and Richardson, T.L. 2011. Extraction protocols for the quantification of phycobilins in aqueous phytoplankton extracts. Journal of Applied Phycology, 23:865.

**ACKNOWLEDGEMENTS:**

We wish to acknowledge the support of all those who helped plan and conduct the verification test, analyze the data, and prepare this report. In particular, we would like to thank our Technical Advisory Committee, Dr. Brian Bergamaschi, U.S. Geological Survey, Dr. Thomas Bridgeman, University of Toledo, Dr. Christopher Gobler, Stony Brook University, Dr. Mary Jane Perry, University of Maine, and Dr. Alan Wilson, Auburn University for their advice and direct participation in various aspects of this evaluation. Earle Buckley also provided critical input on all aspects of this work and served as the independent Quality Assurance Manager. This work has been coordinated with, and funded by, the National Oceanic and Atmospheric Administration, Integrated Ocean Observing System program.

**March 21, 2019**

**Date**

**Approved By: Dr. Mario Tamburri**
**ACT Executive Director**

**March 21, 2019**

**Date**

**Approved By: Dr. Tom Johengen**
**ACT Chief Scientist**

**March 21, 2019**

**Date**

**Approved By: Dr. Earle Buckley**
**Quality Assurance Supervisor**

Turner Designs would like to thank Alliance for Coastal Technologies (ACT) for their efforts in testing our CyanoFluor Handheld HAB Indicator.

CyanoFluor is configured with dual channel detection for tracking *in vivo* fluorescence from both Chlorophyll and Phycocyanin pigments.

Most of the ACT test trials show a good correlation between pigment (w/v) estimates determined via extraction and their respective fluorescence values as measured by CyanoFluor. These results indicate that CyanoFluor is tracking pigment changes as expected and therefore is able to predict the onset of HABs using Phycocyanin to Chlorophyll ratios.

Turner Designs Inc.
1995 N. 1st Street, San Jose, CA 95112
toll free (877)316-8049 / (408)749-0994 / fax (408)749-0998
www.turnerdesigns.com