Ref. No. [UMCES] CBL 2019-008
ACT VS19-03

PERFORMANCE VERIFICATION STATEMENT
For Turner Designs PhytoFind

TECHNOLOGY TYPE: Multispectral 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.

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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 – 150 km and each included seven isolated tank-exposure comparisons comprising 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 PhytoFind which is an *in situ* Algal Classification tool that distinguishes among algal groups using specific fluorescence signatures. Three optical sensors with preset excitation and emission filters are used to determine the abundance of mixed algal groups: PE-containing algae (e.g. Cryptophytes), PC-containing algae (Cyanobacteria), and the Green-Brown group (all other algae). PhytoFind uses de-convolution algorithms dependent on the fluorescence responses detected to calculate group abundances. A fourth sensor corrects for interference from dissolved organic materials (DOM) thereby increasing accuracy in estimates. Sensors are optimized for rejecting interference from suspended sediments to minimize interference from turbidity. A single instrument was provided for all lab and field testing and tests were conducted under the same configuration, with no attempt to optimize response within a given environment or community composition.

Instrument performance across all lab and field tests based on linear regression of the PhytoFind total chlorophyll estimation against extracted chlorophyll is given below in table 1 along with a summary of successful data returns for each of the tests completed. Overall the PhytoFind over-estimated total chlorophyll in the Lab tests and under-estimated chlorophyll in the field tests. CDOM challenge additions exacerbated the over-estimation since CDOM blanking for the Lab tests was only applied to blank media. There was no known explanation for the very low response in Monterey Bay, whereas the low response for the western Lake Erie underway test was not completely unexpected due to the presence of a significant colonial *Microcystis* bloom which is

known to exhibit a low in situ fluorescence response. Failure of the external battery compartment occurred during both moored field deployments which limited the utility of those test applications.

Table 1. Summary of the PhytoFind total chlorophyll fluorometric response regressed against extracted chlorophyll measurements for each of the laboratory and field tests completed during the ACT technology evaluation along with a summary of potential reference comparisons and actual data returns.

Test	Response Slope	Regression R-squared	# Ref Samples	Instrument OBS	Data Return %	Distance or Duration
LAB Tests						
ML Test 1	1.5	94	8	8	100	1 d
ML Test 2	1.6	97	10	10	100	1 d
ML Test 3	0.78	62	31	31	100	1 d
ML Test 4	2.1	99	19	19	100	1 d
ML Test 5	1.8	84	21	21	100	1 d
GLERL	0.93	73	10	10	100	1 d
Field Test Underway						
SF Bay	0.79	95	16	1263	99	150 km
Monterey Bay	0.21	11	14	1265	99	75 km
WLE	0.52	93	14	2072	100	75 km
Field Test Moored						
Maumee River	-0.61	12	31	222 of 620	36	5 of 13 d
Chesapeake Bay	1.0	51	60	120 of 642	19	5 of 28 d

Algal classification results reported by the PhytoFind were compared graphically against algal group biovolume proportion estimates derived from microscopic counts and established shape formulas. We recognize that biovolume is not a direct equivalent for fluorescence contribution but provided the best proxy of community composition. Microscopic analysis differentiated several more groups than the PhytoFind so the main response to consider was for phycocyanin and phycoerythrin containing groups. The PhytoFind clearly responded to phycobilin-containing communities but with a general tendency to over-predict the proportion of Cyanobacteria during the Lab tests and over-predict the proportion of Mixed or phycoerythrin species in the Field tests. The addition of CDOM as a matrix challenge in the lab tests resulted in an increased classification of the Mixed group. A similar response occurred in the field test when natural CDOM content was likely elevated from riverine inputs. These results indicate the importance of the CDOM blanking procedure and highlight the challenge of applying a single correction at the beginning of a test or field deployment when the matrix is likely to be variable over time.

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

PhytoFind is an *in situ* Algal Classification tool that distinguishes among algal groups using specific fluorescence signatures. Three optical sensors with preset excitation and emission filters are used to determine the abundance of mixed algal groups: PE-containing algae (e.g. Cryptophytes), PC-containing algae (Cyanobacteria), and the Green-Brown group (all other algae).

PhytoFind uses de-convolution algorithms dependent on the fluorescence responses detected to calculate group abundances. A fourth sensor detects and corrects for interference from dissolved organic materials (DOM) thereby increasing accuracy in estimates. Sensors are optimized for rejecting interference from suspended sediments; interference from turbidity is minimal.

PhytoFind is factory-calibrated and field ready; no calibration is required. The durable Delrin plastic housing is resistant to harsh environments and damage caused by fouling organisms. Additional anti-biofouling mechanisms include an integrated wiper and/or attachable copper plate. PhytoFind has a depth rating of 600 meters and comes with a factory-installed temperature and depth sensor. Other accessories such as the high capacity external battery, flow cap, and shade cap are available to

accommodate various sampling modes. PhytoFind can be programmed using a simple Windows™ based graphical user interface (GUI) that allows users to view and capture data using real-time mode, configure the instrument for internal data logging mode, and download data. Data reported include group abundances as percent of total population along with temperature, depth, and chlorophyll (µg/L) concentrations. If desired, these data may be used to calculate chlorophyll concentration estimates for each algal group.

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. At the start of testing at each of the three ACT facilities, instrument output was referenced to defined Basic Blue 3 (BB3) solutions at concentrations levels of 0.05 and 0.5 uM under standard conditions to ensure good working order and consistent operational response (see Table 8). The following text summarizes the test protocols used by ACT for all of the instruments to the evaluation with instrument specific details for the PhytoFind defined as appropriate.

Laboratory Tests

Laboratory tests of response linearity, precision, range, and reliability were conducted at Moss Landing Marine Laboratories (MLML). Instrument response to several individual freshwater and marine cultures was quantified at various concentration levels. Instruments were exposed to mixtures of different phytoplankton assemblages within freshwater or marine media. Lastly, matrix effects of turbidity and dissolved organic carbon were assessed through addition of specified concentrations to mixed algal assemblages. *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 mechanically mixed, 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 known mixtures and concentrations of live cultures added into a background

matrix of filtered deionized water or seawater, supplemented with appropriate salt and nutrient additives (BG11+Si and L1 respectively). Freshwater and seawater were obtained from the MLML aquaculture facility.

Phytoplankton Taxa – Algal cultures came from a variety of sources including the traceable commercial entities 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 cyanobacterium (*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 cyanobacterium (*Synechococcus* spp.). Cultures were grown in large 20L batch cultures under cool LED light (ca 75 µmol quanta m^{-2} s^{-1}; 6-8 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 change. 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, given that the ratios are based on pigment content. All additions and test conditions were maintained at low ambient light (< 75 umol photon m^{-2} s^{-1}). Individual algal species were added sequentially to produce different ratios and concentrations. The exact CHL concentrations tested varied depending 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 sequential additions 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 concentration over 15minute exposures, after which, they were challenged with the CDOM and/or turbidity additions. 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 ensure 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. For clarity, 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, the PhytoFind was 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 to 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 were 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 dock-side 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.

Instrument Setup - Prior to deployment, all instruments were setup according to the recommendations and training by the manufacturer. The instruments were tested as supplied and no calibration procedures were applied by ACT staff. 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 20cm 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 work week, 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 incubate 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's fixed sample aliquots. A whole water subsample was collected to measure turbidity using a Hach model2100AN 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 6th 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 13th 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 13th 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 - For the underway surface mapping test instruments were programmed to record data at one 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 extractive 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; 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, typically controlled at 20 ± 1.0 ^{o}C.

A total of six replicates were 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, poly-carbonate centrifuge tubes. Pigment analysis was conducted on two replicates for chlorophyll and three replicates for

phycobilins. One filter was reserved in storage at -80 ºC and subsequently analyzed when the variability in the initial results were above a threshold of 20% in coefficient of variation. All reference sample pigment analyses were 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 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 followed 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 um 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 1nm 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. The CDOM blanking procedure was applied before each lab test using only culture media and did not account for CDOM addition as part of a matrix challenge.

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 2 – 6, 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 2).

Table 2. 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 concentrations 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 test batch including one immediately after sample preparation and the second at the end of all instrument exposures. Results are plotted as a time series of instrument readings compared to extracted pigment concentrations in µg/L determined on the reference samples (Figure 1). The absolute difference between instrument estimation and reference measurement increased at the higher concentration levels. There was no apparent difference in accuracy across the two species, although this could not be tested statistically given the experimental design.

A cross plot of PhytoFind readings compared to reference sample measurements for CHL is shown in figure 2. The regression line for the CHL response over the tested range of 0 to 32 µg/L was statistically significant ($p=0.03$) with an R^2 of 0.94 and a slope of 1.46, resulting in over-predictions by the PhytoFind at higher concentrations.

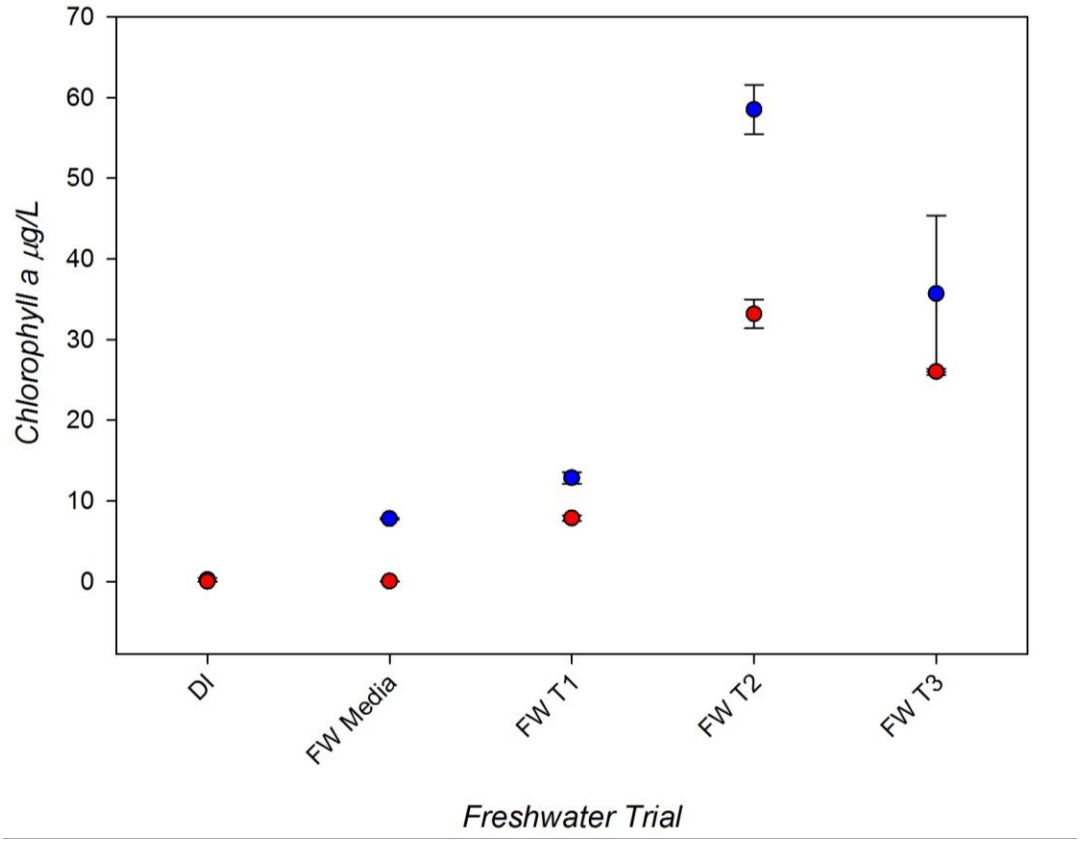


Figure 1. Plot of instrument (blue) and reference (red) measurements of chlorophyll a in the freshwater individual algae tests, including background readings for DI and the freshwater culture media. Two replicate reference measurements were made from subsamples taken at the beginning and end of exposure. Instrument estimations were generated from the 10-second readings averaged over 2 minutes, following an equilibration time of 5 minutes.

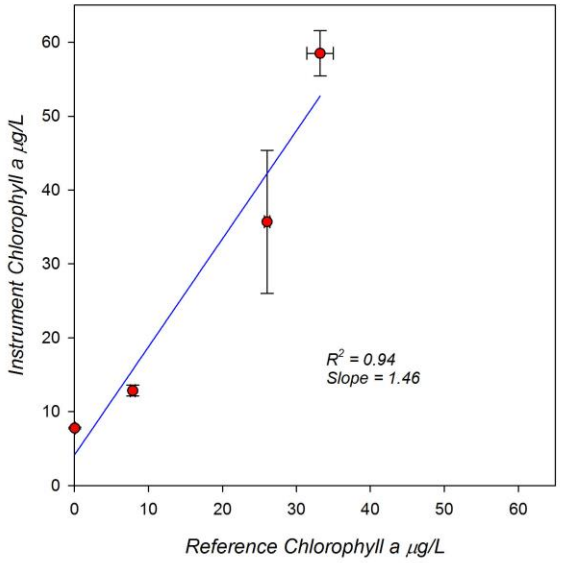


Figure 2. Cross plot of instrument readings versus extracted reference sample pigments for chlorophyll a during the freshwater individual algae lab trials. The blue line represents the linear regression of the data.

A comparison of algal classification from microscope counts on preserved reference samples against the PhytoFind classifications is shown in figure 3. We do note that some contamination of *Microcystis* in the *Coelastrum* culture was discovered in subsequent trials (and noted by phycocyanin measurements) but was not detected in the microscopy analysis of the reference sub-samples counted. The PhytoFind did not accurately distinguish between green algae and cyanobacteria during the *Microcystis* culture exposure in trial 3.

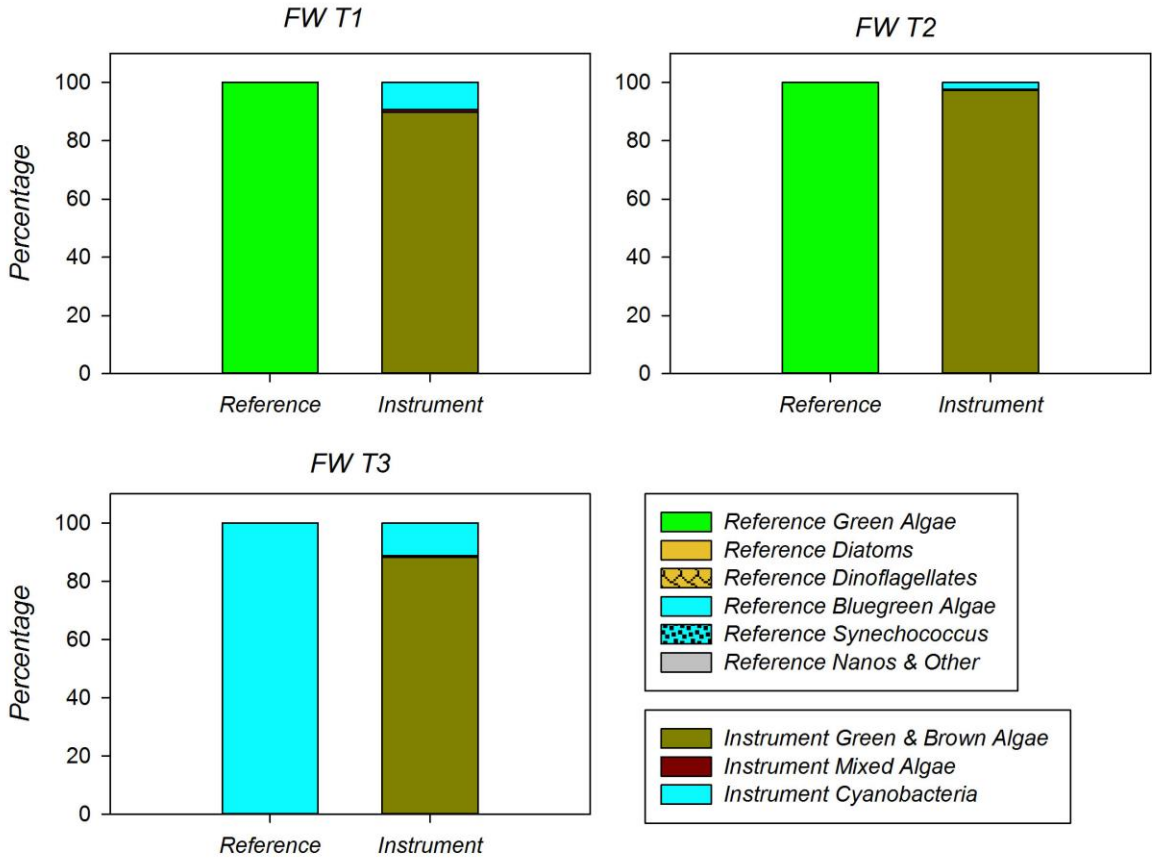


Figure 3. Algal classification from microscope counts on preserved reference samples against the PhytoFind classifications as a percentage of biomass, with instrument algae categories in percentage of total chlorophyll a. Algal counts were grouped at the functional class level.

Four individual trials were conducted using saltwater algal cultures along with the DI and saltwater media blanks (Table 3). Trials 1 and 2 used two different levels of the golden-brown haptophyte *Isochrysis* at approximately 5 and 22 µg/L CHL, respectively. Trials 3 and 4 used two different levels of the dinoflagellate *Amphidinium* added at concentrations of approximately 5 and 20 µg/L CHL, respectively. 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 3. 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 of instrument readings compared to extracted pigment concentrations determined on the reference samples are plotted in figure 4. The PhytoFind showed low background response to the seawater media and tracked chlorophyll levels more accurately at the lower concentrations for both species than during the second, higher concentration additions. There was no apparent difference in response across the two algal species tested but this was not examined statistically given the experimental design.

A one-to-one cross plot of the PhytoFind chlorophyll estimations compared to reference sample CHL measurements during the saltwater trials is shown in figure 5. The regression line for the CHL response was highly significant ($p=0.002$) with an R^2 of 0.97 and a slope 1.63, so again the instrument over-predicted concentrations based on the current calibration settings as tested.

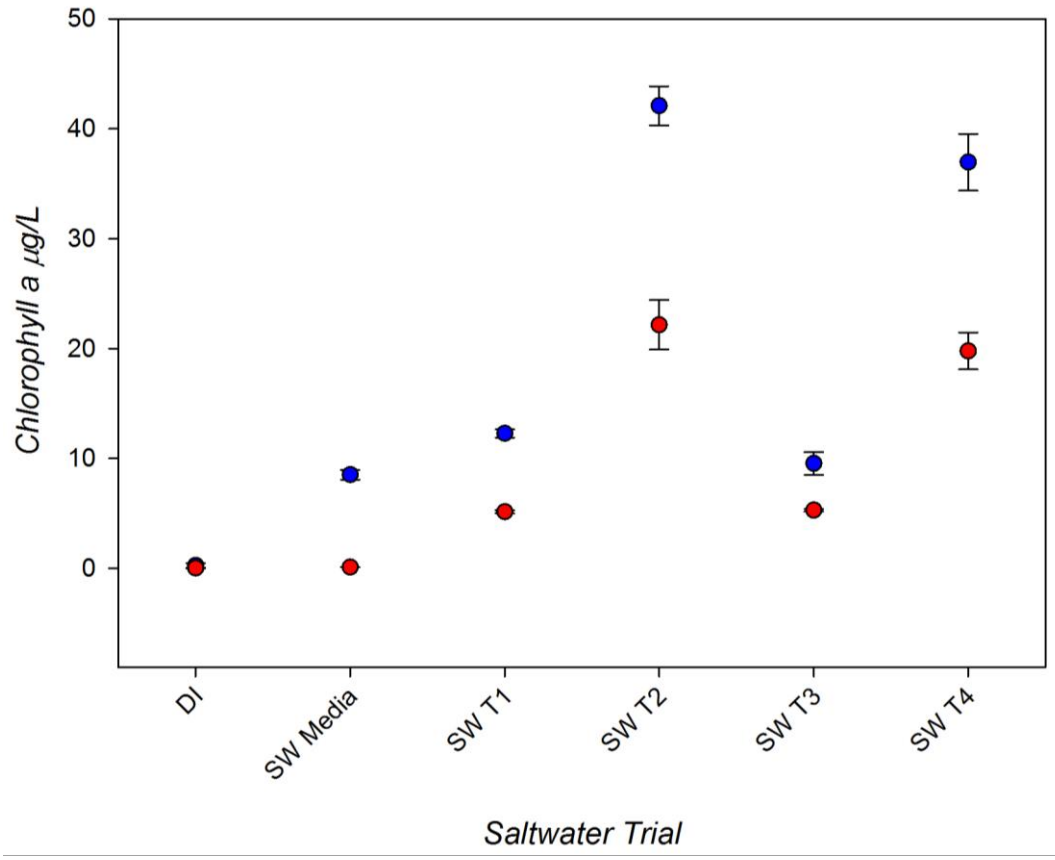


Figure 4. Plot of instrument (blue) and reference (red) CHL measurements in the saltwater individual algae tests including readings for DI and saltwater media. Plotted reference values represent the average and standard deviation of the two reference measurements taken at the beginning and end of the exposure period. Instrument estimations were generated from the 3 second readings averaged over 1 minute, following an equilibration time of 3 minutes.

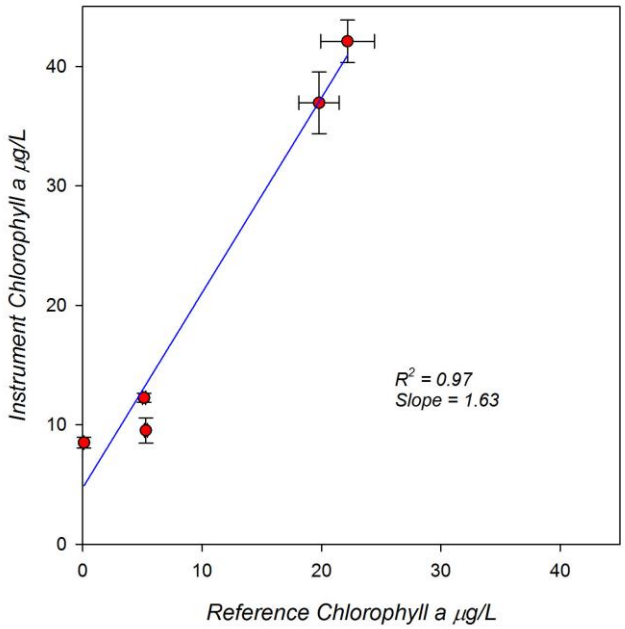


Figure 5. Cross plot of instrument readings versus extracted reference sample pigments for chlorophyll-a during the saltwater individual algae lab trials. The blue line represents the linear regression of the data.

A comparison of algal classification from microscope counts on preserved reference samples against the PhytoFind classifications is shown in figure 6. The PhytoFind largely classified the cultured samples in the green-brown category as expected, but also called out contributions from cyanobacteria and mixed species for trials 1 and 3. We recognize that some contamination may have been present that was not picked up in our microscopic analysis for these cultures, but unlikely at the percentages indicated. No phycobilins were detected in reference samples from these trials. It is not known why the classifications were not consistent across the two concentrations of the same culture addition (i.e., between T1 and T2 and between T3 and T4).

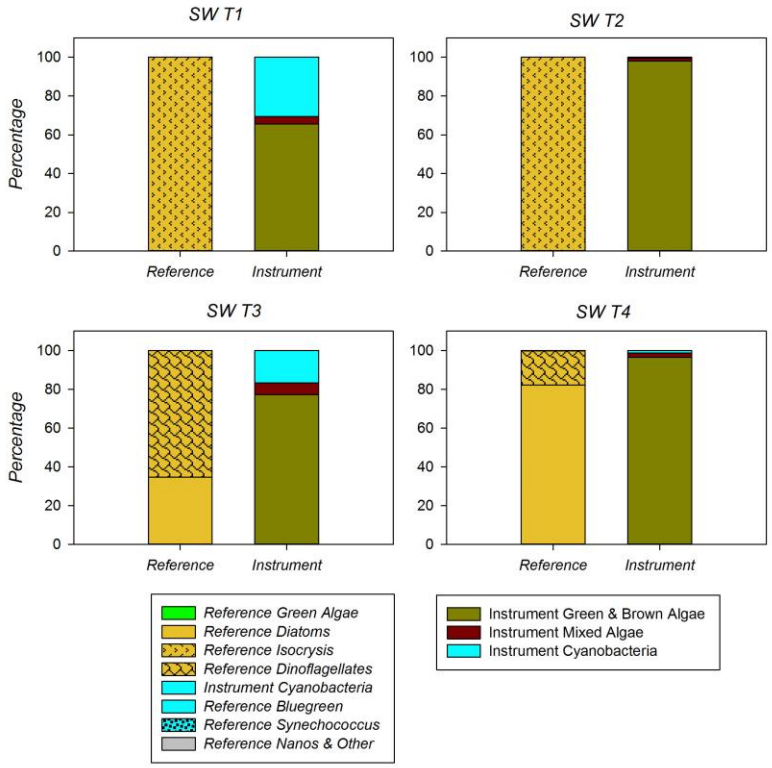


Figure 6. Algal classification from microscope counts on preserved reference samples against the PhytoFind classifications as a percentage of biomass with instrument algae categories in percentage of total chlorophyll a. Algal counts were grouped at the functional class level. Estimations of Isocrysis in trials 1 and 2 were based on examination of the culture and known culture addition volumes and not directly confirmed with microscopic counts on the reference sample aliquots.

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 4). It should be noted that the CDOM blanking procedure was only applied to the seawater media at the beginning of the test. 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. Comparative PhytoFind results were generated from 3 minute averages bracketing each reference point. 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 4. June 27 test conditions with exposures to combinations of saltwater algal cultures at various concentrations with add-in challenges of turbidity and CDOM. (n = reference samples collected during the exposure and the values in parenthesis show averaged concentrations determined on the reference samples).

Trial	*Synechococcus*	CDOM (A_{400})	Turbidity (NTU)
SW Media (n = 1)		(0.25)	(0.69)
SW T1 (n = 3)	C1 (3.2)	(0.25)	(0.55)
SW T2 (n = 3)	C2 (6.4)	(0.24)	(0.54)
SW T3 (n = 3)	C2 (6.1)	C1 (0.83)	(0.34)
SW T4 (n = 3)	C2 (6.2)	C2 (1.76)	(0.48)
SW T5 (n = 3)	C2 (6.0)	C3 (3.25)	(0.44)
SW T6 (n = 3)	C3 (19)	C3 (3.05)	(0.65)
SW T7 (n = 3)	C4 (38)	C3 (2.86)	(1.0)
SW T8 (n = 3)	C4 (40)	C3 (2.90)	C1 (3.3)
SW T9 (n = 3)	C4 (37)	C3 (2.90)	C2 (23)
SW T10 (n = 3)	C4 (36)	C3 (2.97)	C3 (50)

Over the ten algal trials, reference sample CHL levels ranged from 3.2 to 40 µg/L and PC levels ranged from 0 to 1.5 µg/L. Across the trials, CDOM concentrations were increased from a background level of approximately 0.25 up to 3.0, and turbidity was increased from a background level of 0.5 up to 50 NTU.

Comparative results of instrument readings versus reference sample CHL concentrations in µg/L are plotted in figure 7. The PhytoFind showed low background response to the seawater media and overall tracked the chlorophyll additions, however, there was also a strong positive bias to CDOM additions as seen by the greater offset during trials 3, 4, and 5. This result was expected since CDOM blanking was only applied using the seawater media which had almost no CDOM. There did not appear to be any strong bias in the response during the turbidity additions (trials 8-10).

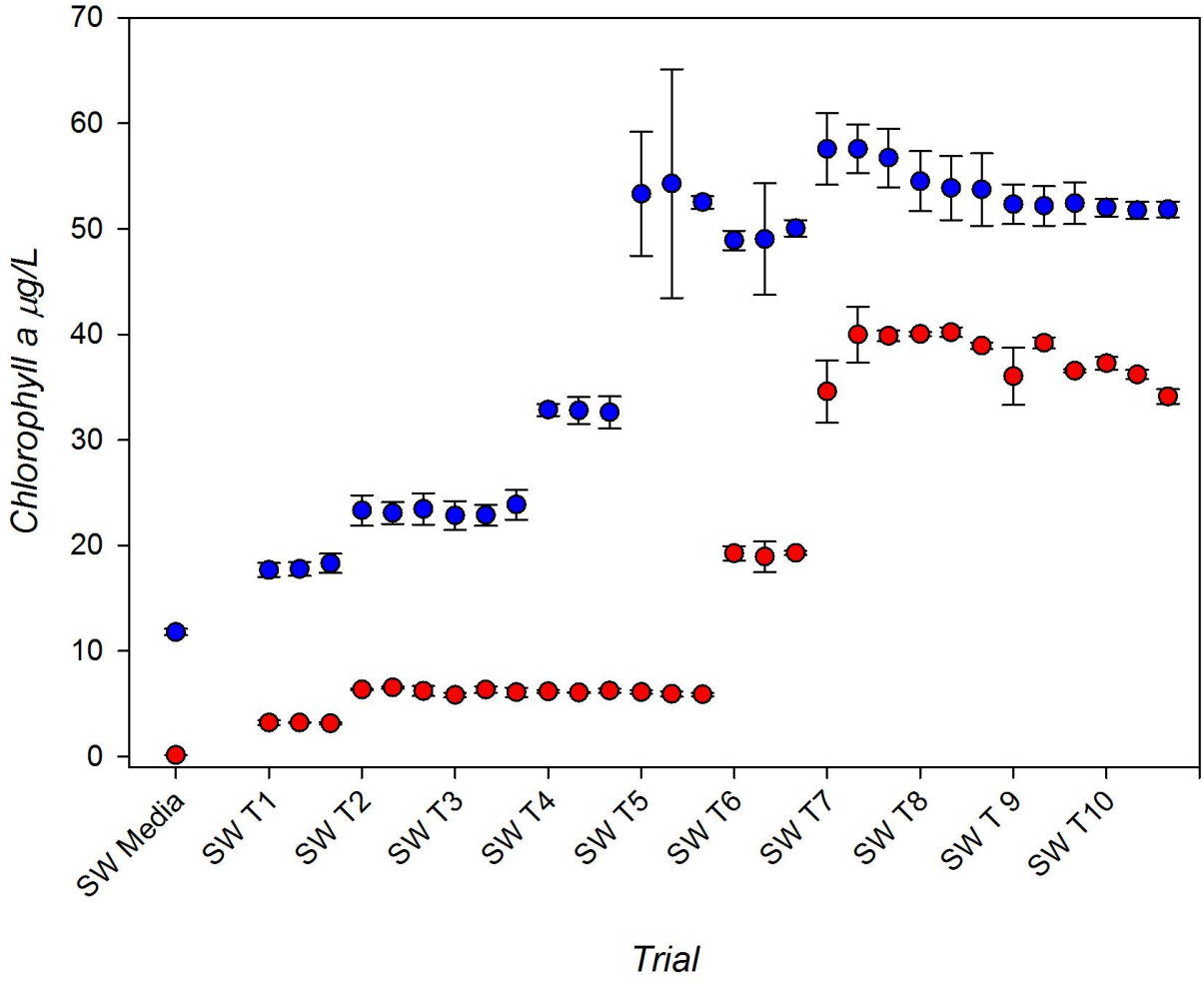


Figure 7. Plot of instrument (blue) and reference (red) measurements of chlorophyll a in the saltwater CDOM and turbidity addition trials covering 4 algae, 3 CDOM and 3 turbidity concentration levels. Three replicate reference measurements were made at each level with only one read in blank media. PhytoFind data were averaged over 3 minutes, bracketing each reference point.

A one-to-one cross plot of the PhytoFind readings compared to reference sample measurements for CHL during the saltwater trials is shown in figure 8. The regression line for the CHL response was highly significant ($p<0.001$) with an R^2 of 0.62 and a slope of 0.78. This response slope is noticeably lower than for the previous two lab tests and the variation in response is clearly noticed for the trials with CDOM additions centered around a reference CHL concentration of 6.2 µg/L. Again, CDOM blanking with an appropriate sample matrix is necessary to provide a more accurate CHL estimation.

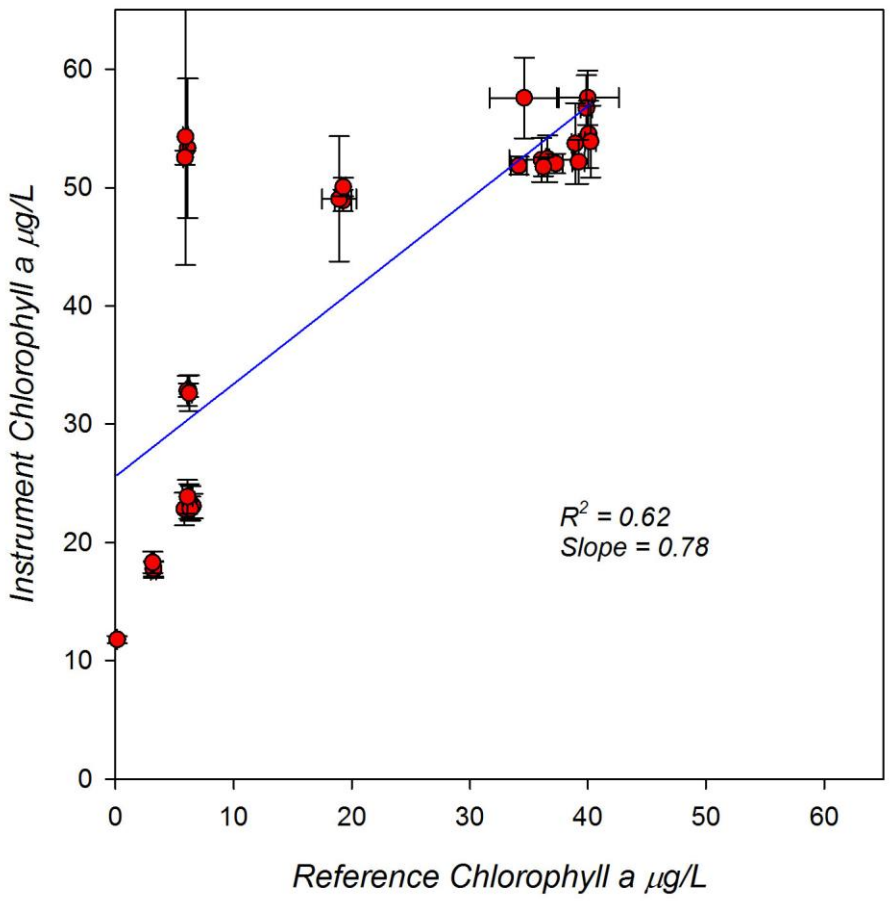


Figure 8. Cross plot of instrument readings versus extracted reference sample pigments for chlorophyll during the saltwater *Synechococcus* algae exposure with add in matrix challenges for CDOM and turbidity. The blue line represents the linear regression of the data.

A comparison of algal classification from microscope counts on preserved reference samples against the PhytoFind classifications for the *Synechococcus* with CDOM and turbidity additions is shown in figure 9. The PhytoFind classifications were consistent across the three timepoint replicates (reps a,b,c within a row) for each of the trials. For trial 1, (*Synechococcus* only) the PhytoFind classified the test solution as roughly 20% Green-Brown and 80% cyanobacteria. As more culture was added in trial 2 PhytoFind increased the Green-Brown classification to 40%, consistent with counts showing more diatoms and less *Synechococcus.* With the addition of CDOM starting in trial 3, the PhytoFind classified from 25 to 70% of the fluorescence response as Mixed Algae in direct proportion to the amount of CDOM added. It must be noted for these Lab test no CDOM blank correction was applied to the instrument, and we did not try to assess how effective the blanking procedure would be for improving classification accuracy. The PhytoFind did continue to classify about 25% of the fluorescence to Cyanobacteria which likely reflected the prescence *Synechococcus, although by bivolume it was at a much lower percent.* There was no obvious influence from the addition of turbidity on the classification estimations.

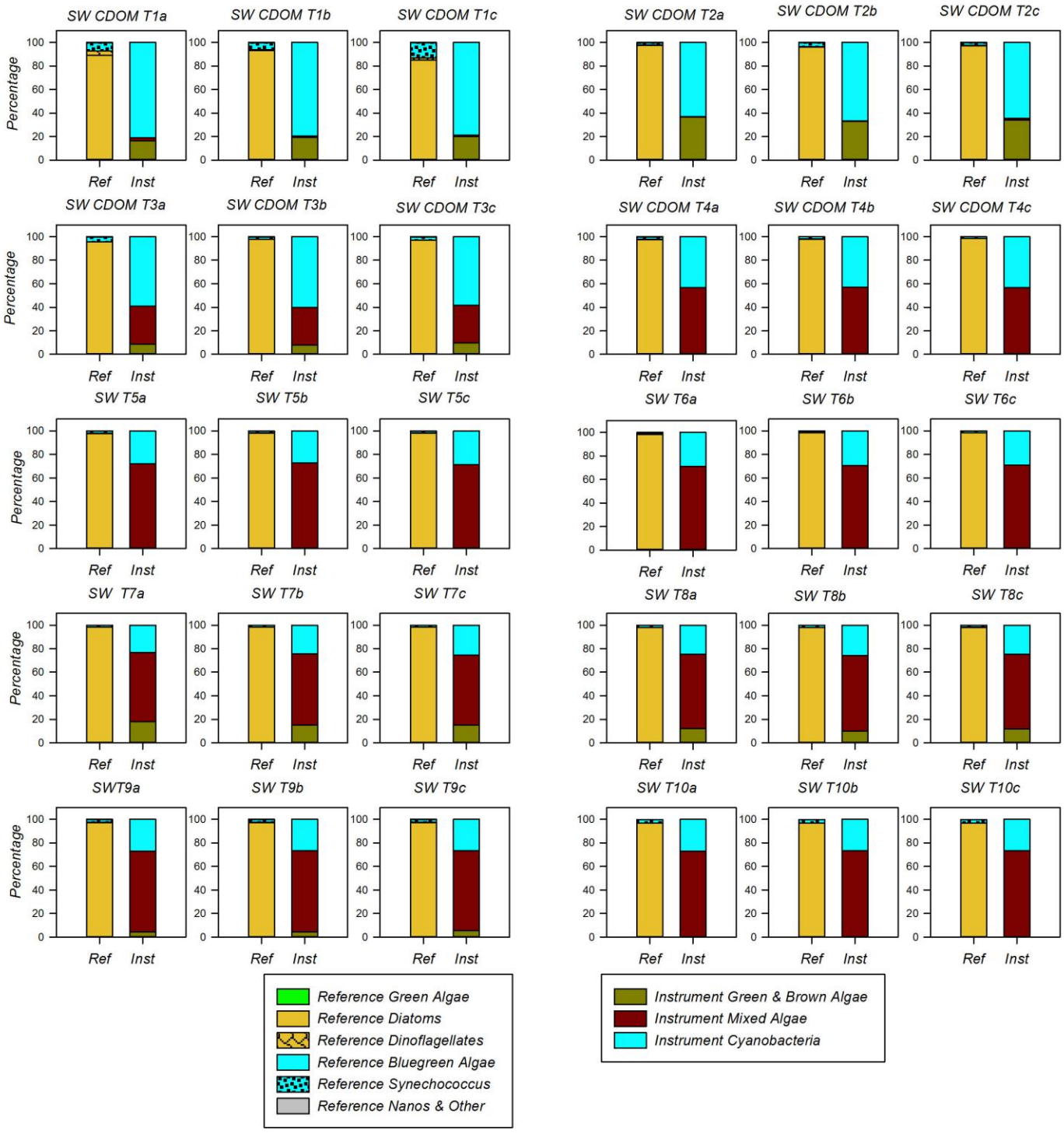


Figure 9. Reference cell counts as a percentage of biomass compared to instrument algae categories as a percentage of total chlorophyll a. Each row represents three timepoint replicates (a, b, c) of the same batch of algae and matrix conditions.

For the June 28th lab test, 6 different trials were conducted using various mixtures of *Coelastrum*, *Microcystis* and *Peridinium* at varying concentrations, along with a blank for the freshwater media (Table 5). 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. 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 5. June 28 test conditions with exposures to combinations of freshwater algae at various concentrations. (n = number of reference samples taken during the exposure; C1 and C2 represent concentrations levels from culture additions).

Trial	*Coelastrum*	*Microcystis*	*Peridinium*
FW Media (n = 1)	-	-	-
FW T1 (n = 3)	C1	-	-
FW T2 (n = 3)	C2	-	-
FW T3 (n = 3)	C2	C1	-
FW T4 (n = 3)	C2	C2	-
FW T5 (n = 3)	C2	C3	-
FW T6 (n = 3)	C2	C3	C1

Comparative results of instrument readings versus reference sample CHL concentrations in µg/L for the June 28 lab test are plotted in figure 10. The PhytoFind showed low background response to the freshwater media and generally tracked chorophyll levels across all mixtures of the three algal groups over the test range from 4 to 25 µg/L, but with a slope significantly greater than one. Therefore the magnitude of measurement difference against the reference samples was proportional to the concentration.

A one-to-one cross plots of the PhytoFind readings compared to reference sample measurements for CHL during the freshwater algal mixture trials are shown in figure 11. The regression line was highly significant ($p<0.001$) with an R^2 of 0.99 and a slope of 2.12, resulting in over estimations of CHL at higher concentrations.

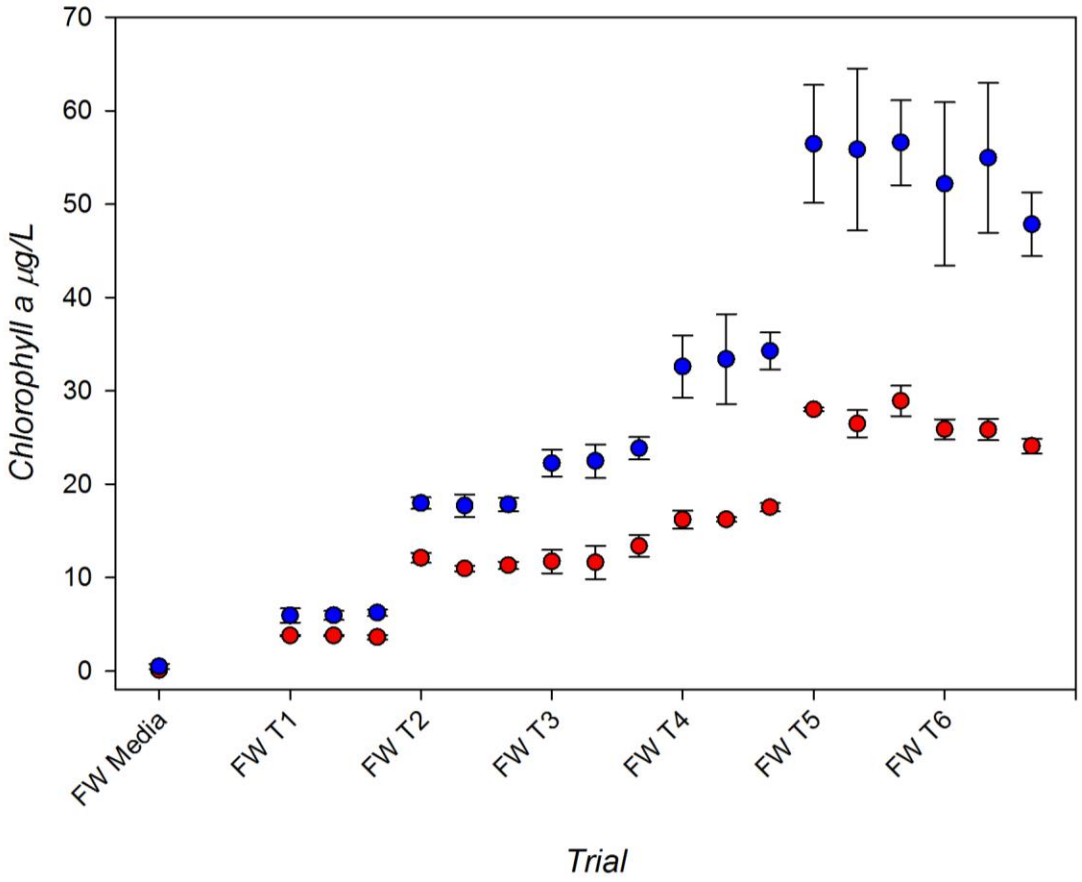


Figure 10. Plot of instrument (blue) and reference (red) measurements of chlorophyll a in the freshwater algal mixture trials covering 6 concentration ranges and mixtures of 3 different algae. Three replicate reference measurements were made at each level, and the PhytoFind data were averaged over the 2 minutes bracketing each reference point.

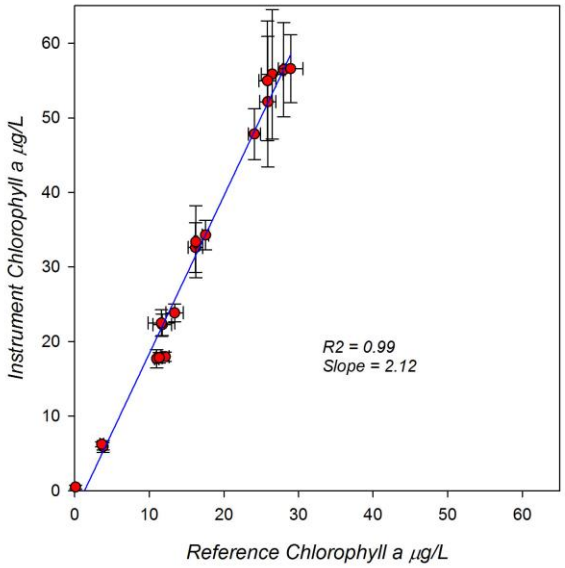


Figure 11. Cross plot of instrument readings versus extracted reference sample pigments for chlorophyll a during the June 28 freshwater algal mixtures lab trials. The blue line representsthe linear regression of the data.

A comparison of algal classification from microscope counts on preserved reference samples against the PhytoFind classifications for the freshwater algal mixtures is shown in figure 12. In the first two trials, the PhytoFind attributed approximately 20% of the fluorescent signal to Cyanobacteria and Mixed species. We do recognize that the *Coelastrum* culture was not pure even though it was not detected in our reference sample counts. Although we saw no measurable PC in trial 1, we did find about 1 µg/L PC in trial 2 with the higher addition of *Coelastrum* cells. In subsequent trials 3-6 with addition of *Microcystis*, the PhytoFind called out a consistent Cyanobacteria contribution, and the percent contribution did not vary across the trials even though the abundance of *Microcystis* was increased by a factor of seven at the highest levels (based on extracted PC and volumetric addition to the test tank).

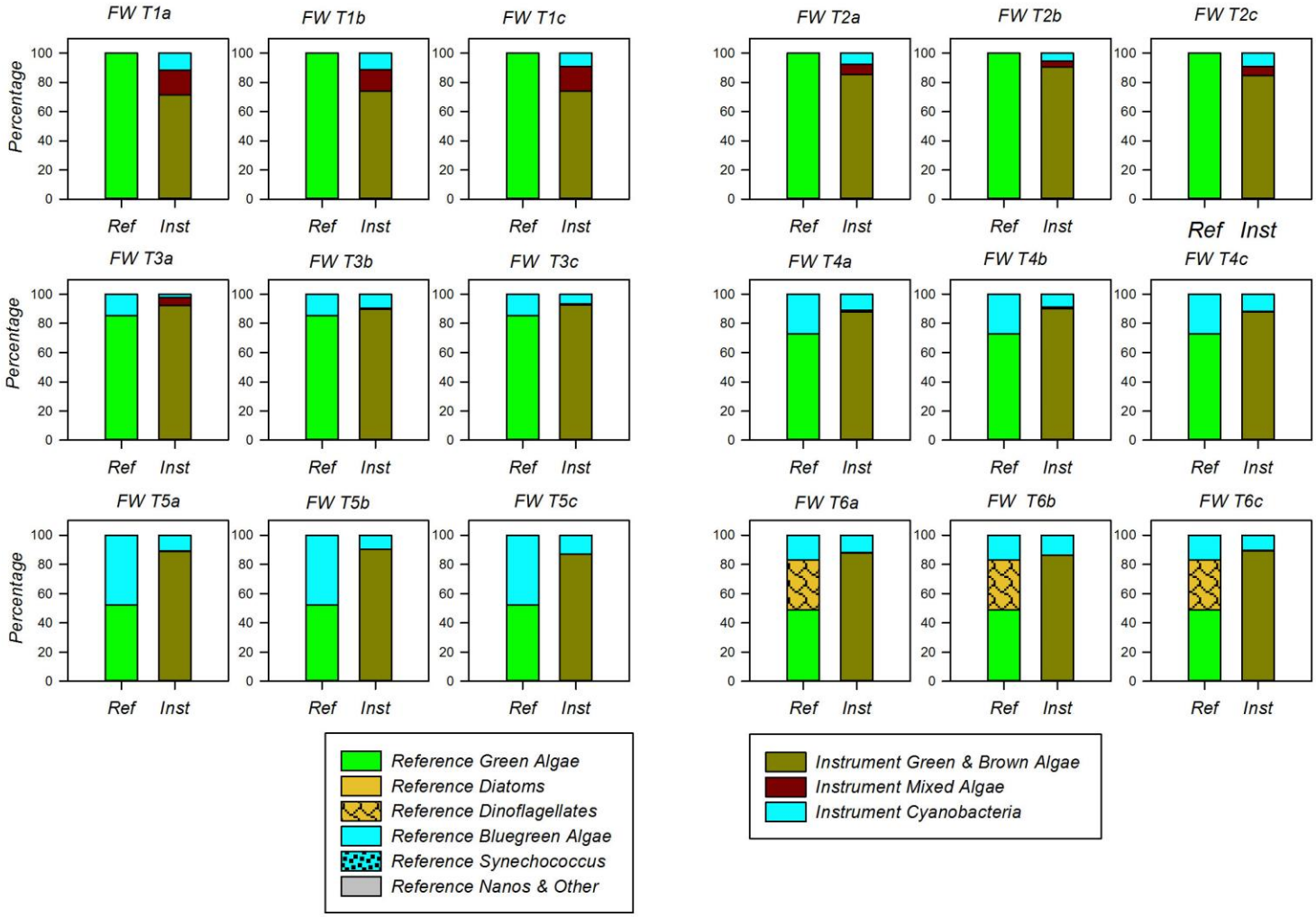


Figure 12. Reference cell counts as a percentage of biomass compared to PhytoFind portioning of algal categories as a percentage of total chlorophyll a.

For the June 29 lab test, eight different trials (plus a media blank) were conducted using three levels of *Microcystis* with add-in matrix challenges of three CDOM levels, an addition of *Coelastrum*, and lastly an addition of turbidity (Table 6). It should be noted that the CDOM blanking procedure was only applied to the freshwater media at the beginning of the test. 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. Over the eight algae 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 level of 0.43 up to 4.7, and the turbidity additions increased concentrations from a background of 1 NTU up to 25 NTU.

Table 6. June 29 test conditions with exposures to combinations of freshwater algal cultures at various concentrations with add-in challenges of turbidity and CDOM (n = number of reference samples taken during the exposure; C# denotes increasing concentration from additional culture; and measured concentrations of CDOM and turbidity for reference samples are provided in parenthesis).

Trial	*Microcystis*	CDOM (A_{400})	*Coelastrum*	Turbidity (NTU)
FW Media (n = 1)	-	-	-	-
FW T1 (n = 3)	C1	(0.43)	-	(0.25)
FW T2 (n = 3)	C2	(0.43)	-	(0.39)
FW T3 (n = 3)	C2	C1 (1.5)	-	(0.38)
FW T4 (n = 3)	C2	C2 (3.3)	-	(0.45)
FW T5 (n = 2)	C2	C3 (6.4)	-	(0.56)
FW T6 (n = 2)	C3	C3 (4.6)	-	(0.86)
FW T7 (n = 2)	C3	C3 (4.6)	C1	(1.1)
FW T8 (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 compared to reference sample CHL in µg/L (Figure 13). As was seen during the June 27 lab test, the PhytoFind showed low background response and overall tracked the chlorophyll additions but with a response slope significantly greater than 1. In this test there was also a strong positive bias to CDOM additions as seen by the greater offset during trials 3, 4, and 5 when chlorophyll concentration was held relatively constant. There did not appear to be any strong bias in the response during the turbidity additions (trial 8). There was significant variation in the two reference sample CHL estimates for trial 8 (grey symbol), but not for the PhytoFind. Cell counts in replicate two for trial 8 were also 25% lower so suggest some patchiness in the tank during the sub-sampling, but should not be interpreted as an inaccurate reading by the PhytoFind for the timepoints within that trial.

A one-to-one cross plot of the PhytoFind readings compared to reference sample measurements for CHL during the freshwater algal mixture trials are shown in figure 14, with the suspect reference measurement omitted. The regression line was highly significant ($p<0.001$) with an R^2 of 0.84 and a slope of 1.81. Not blanking for CDOM likely contributed to the higher response slope as well.

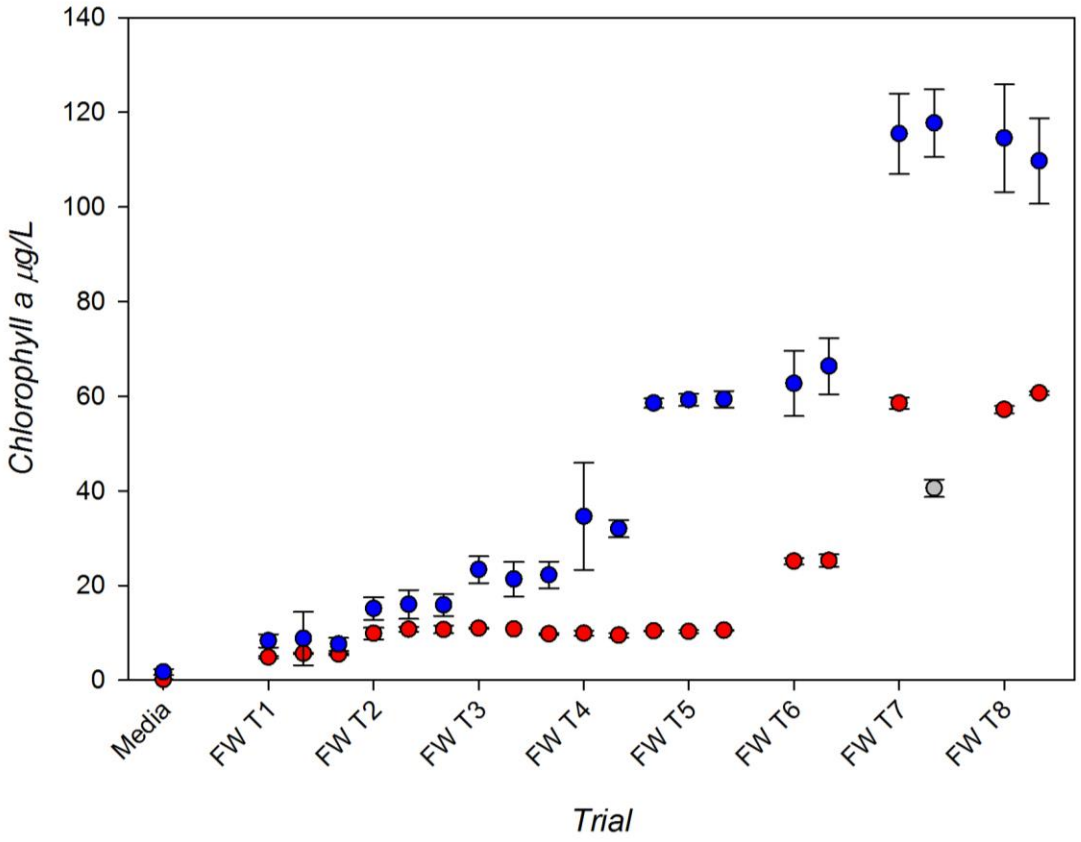


Figure 13. Plot of instrument (blue) and reference (red) measurements of chlorophyll a during the June 29th laboratory test with the freshwater algae *Microcystis* and *Coelastrum*, and challenge additions of CDOM and turbidity. Three replicate reference measurements were made for trials 1-5, two replicates were made for trials 6-8, and one replicate for the blank media. PhytoFind data were averaged over the 3 minutes bracketing each reference sample.

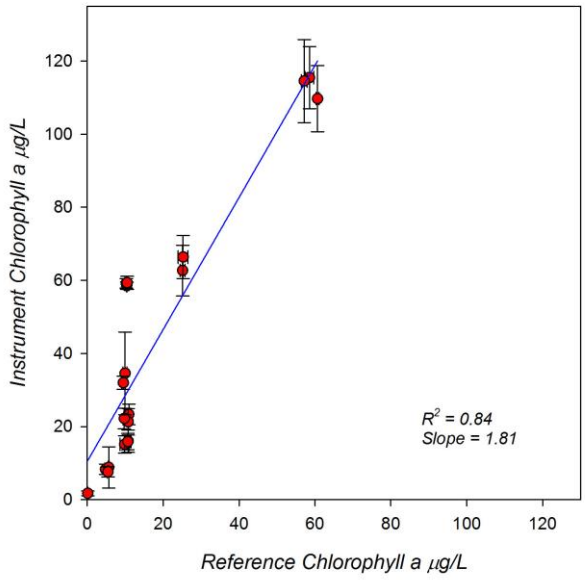


Figure 14. Response plot for PhytoFind chlorophyll a compared to reference samples for the freshwater algae addition lab trials. The blue line represents the linear regression of the data.

A comparison of algal classification from microscope counts on preserved reference samples against the PhytoFind classifications for the freshwater algal mixtures is shown in figure 15. For trials 1 and 2, (*Microcystis* only) the PhytoFind assigned approximately 70 % of the fluorescence signal to the Green-Brown classification. When CDOM was added to the *Microcystis* culture in trial 3 the PhytoFind classified about 40% of the fluorescence to the Mixed category. When CDOM was increased in trials 4 and 5, the PhytoFind proportionately classified a greater percentage of the fluorescence to the Mixed category. Again we note CDOM blanking was only done on the media which contained almost no CDOM. In trial 6, additional *Microcystis* was added but the PhytoFind assigned this contribution to the Green-Brown algae category. The PhytoFind more accurately characterized the addition of *Coelastrum* into the matrix during trials 7 and 8.

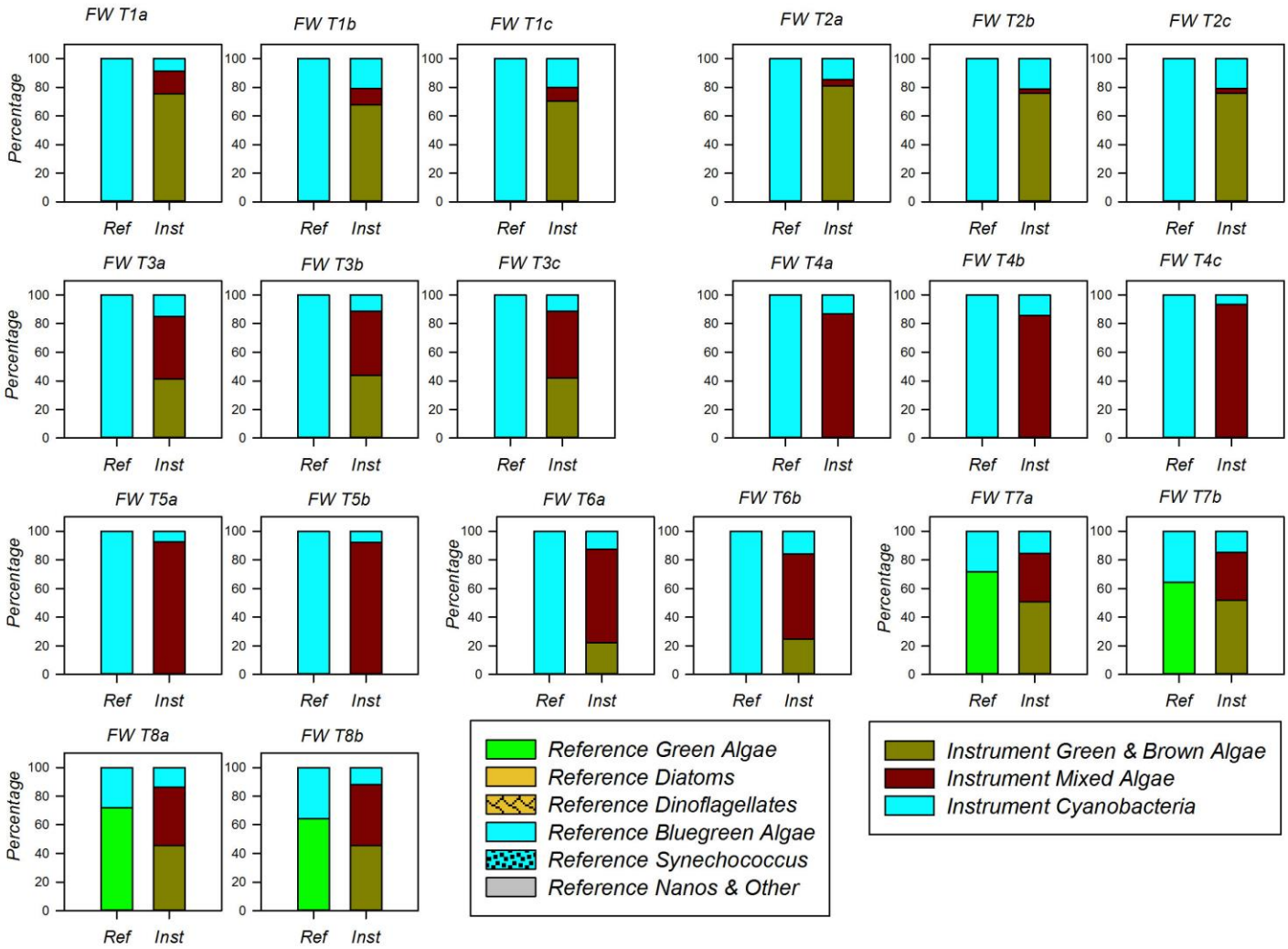


Figure 15. Reference cell counts as a percentage of biomass compared to instrument algae categories as a percentage of total chlorophyll-a.

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 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 7. The next four trials used a mixture of all four algal species to access 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 composition as the preceding trial. CDOM blanking was applied only using the freshwater media. 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. PhytoFind results are taken as an average of 10 second readings around the 5th through 7th minutes of exposure.

Table 7. Great Lakes lab test conditions 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 which was 20% of C2. Reference sample CHL concentrations in µg/L for each culture addition (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 for this lab test are plotted (Figure 16) as a time series of instrument measurements compared to extracted chlorophyll a concentrations in µg/L determined in the reference samples. Overall, the PhytoFind tracked the CHL concentration over the tested range from 12 to 66 µg/L, however the accuracy of predicted CHL concentrations was species dependent. The PhytoFind had a much greater over-estimation of CHL for the *Chlorella* and *Cryptomonas* cultures relative to the *Aphanizomenon* and *Peridinium* cultures. The response to the mixtures was therefore dependent on the proportions of these species. Individual response curves for the monocultures were not established to accurately quantify these biases.

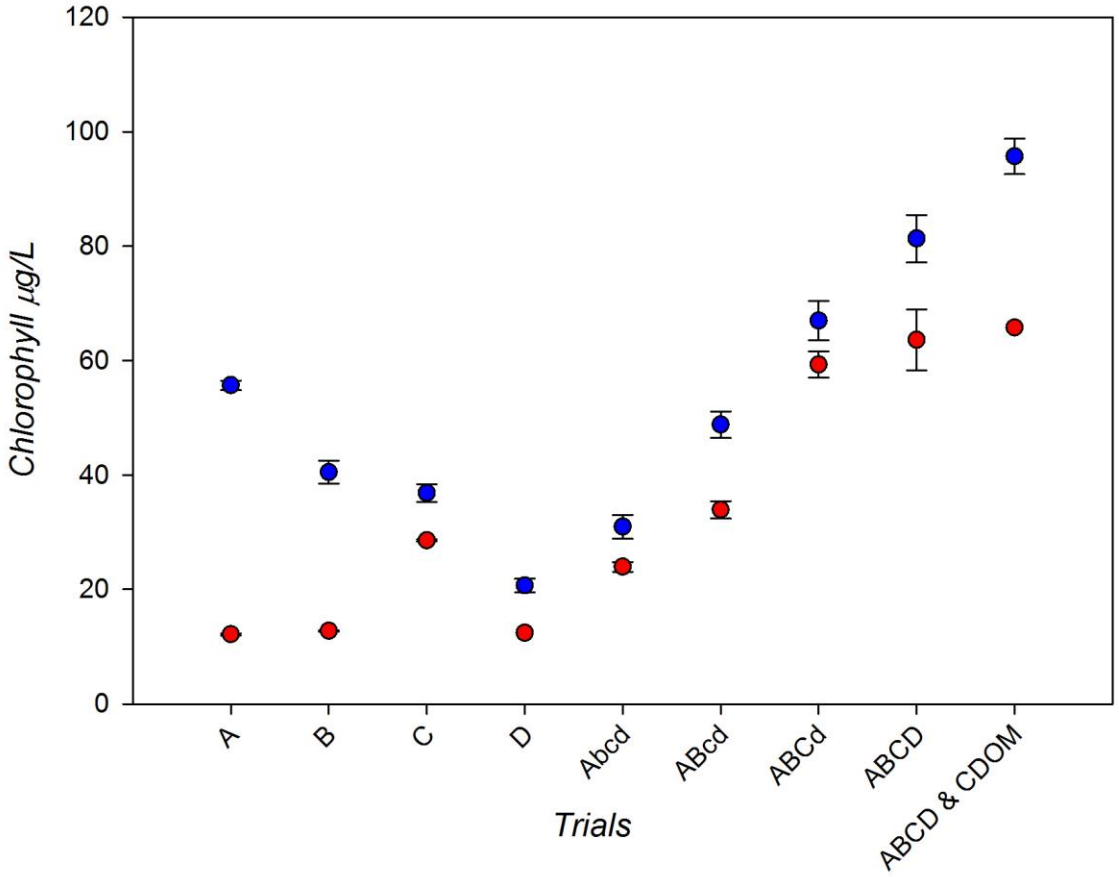


Figure 16. Plot of instrument (blue) and reference (red) measurements of chlorophyll a in the Great Lakes laboratory trial over 4 individual algae cultures, 4 mixtures and a CDOM and turbidity addition. One reference measurement was made at each level and instrument measurements were averaged over 5 minute periods.

A one-to-one cross plot of the PhytoFind readings compared to reference sample measurements during the freshwater algal mixture trials is shown in figure 17. The regression line was highly significant ($p=0.003$) with an R^2 of 0.73 and a slope of 0.93. The greater variation in instrument response at the lowest concentration was due to measurement differences among specific algal species and to the addition of CDOM at the last trial.

A comparison of algal classification from microscope counts on preserved reference samples against the PhytoFind classifications for this freshwater lab test is shown in figure 18. The PhytoFind over-reported the contribution of cyanobacteria in the pure *Chlorella* and *Peridinium* cultures and for the *Cryptomonas* culture attributed about 50% to PC signatures, 10% to PE signatures, and 40% to CHL signatures. The classification percentages become relatively more accurate within the mixtures but with a positive bias of assigning cyanobacteria when *Cryptomonas* was present. The addition of CDOM produced a strong positivie bias in the Mixed Algae classification as was noted in the earlier lab trials.

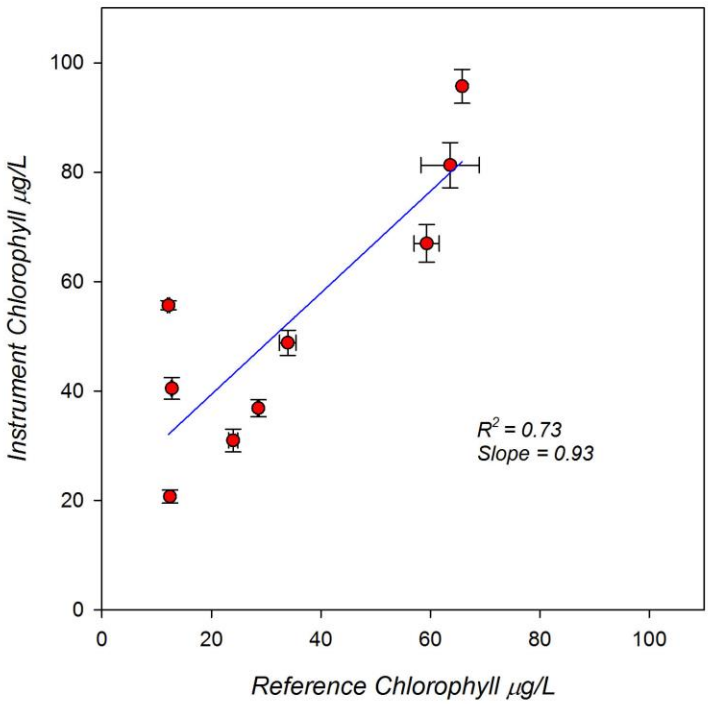


Figure 17. Response plot for the Great Lakes lab test of the PhytoFind compared to reference samples. The instrument values were obtained by averaging over 5 minutes. The blue line represents the linear regression of the data.

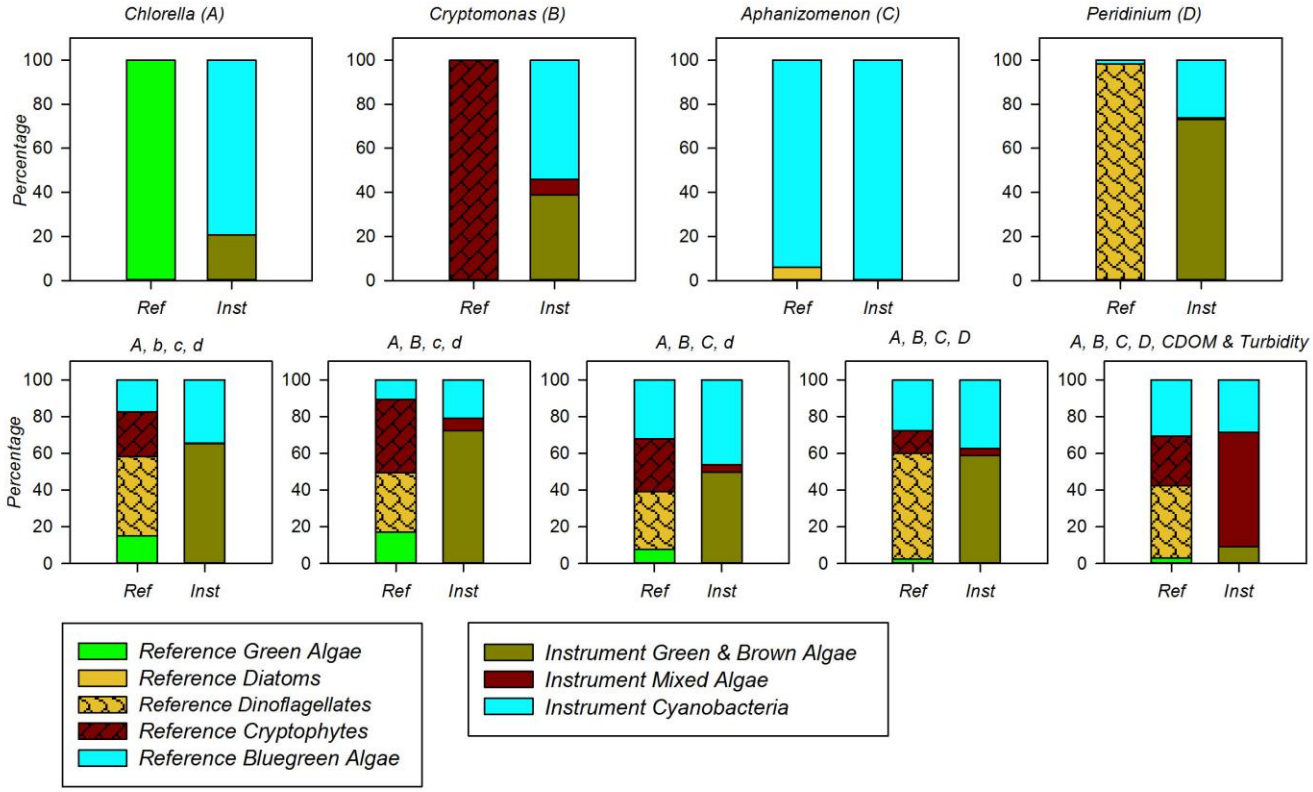


Figure 18. Reference cell counts as a percentage of biomass compared to instrument algae categories as a percentage of total chlorophyll a.

FIELD TESTS

Five field tests were conducted as part of the performance evaluation of the PhytoFind 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 PhytoFind and the consistency of its response over the duration of the evaluation. The CDOM blanking procedure was applied to the PhytoFind using dock-side ambient water before the start of each field test.

Table 8. Results of the pre-deployment DI and BB3 dye check for the PhytoFind for each testing 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

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 at 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 19). During the 150 km underway mapping cruise, eight stations were selected to make comparative reference sample measurements.

R/V David H. Peterson

The *R/V Peterson* 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 attached to valves to control flow rates into the tank. Flow rate was sufficient to fill the tank to overflow within 2 min. Instruments were hung from a PVC frame with sensors oriented toward the bottom of the tank. Coordinated rotation of the rack and instruments was used to clear accumulated bubbles and debris. Port valves were open between station transits to permit continuous turnover of the contained water. The tank lid was kept closed except when mixing and sampling. 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.

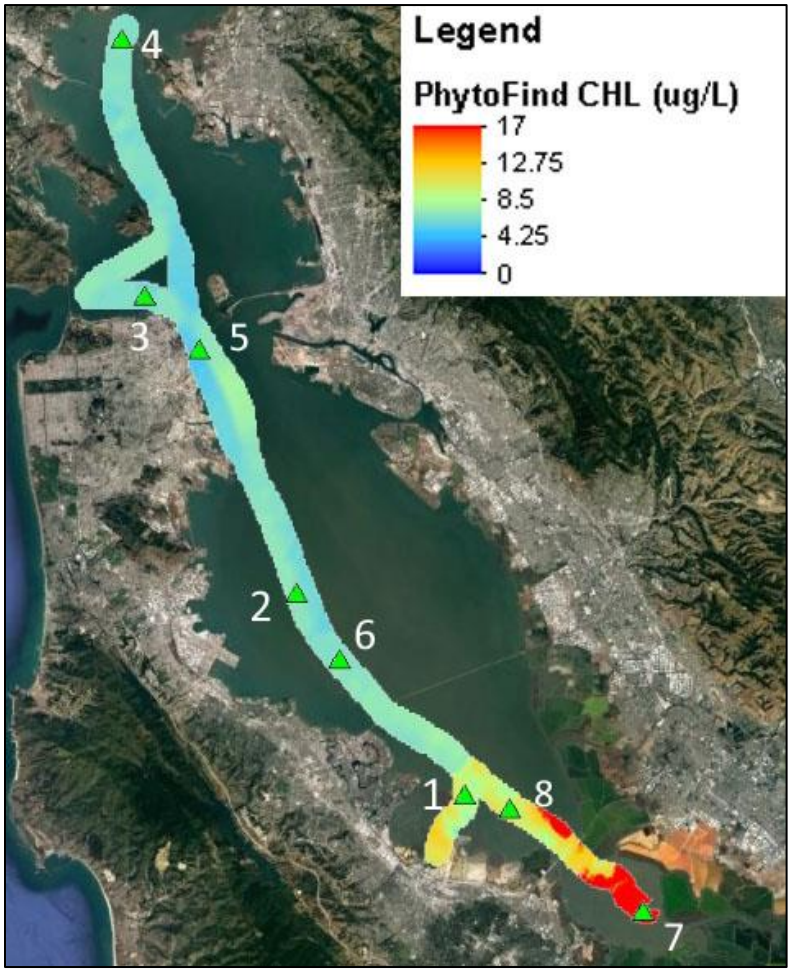


Figure 19. Chlorophyll data contours from the PhytoFind during the underway surface mapping survey in San Francisco Bay on the USGS *R/V Peterson*. Green triangles denote isolated, comparative sampling stations.

During the survey, the PhytoFind produced 1273 readings, of which 10 values were considered outliers for a successful data return of 99% (Figure 19). CHL estimations from the PhytoFind ranged from 4.4 to 20.8 µg/L over the entire survey. Other water quality conditions and descriptions of algal classifications are described below.

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 20). Continuous measurements indicated that conditions in the tank during isolation periods were relatively stable. Reference sample analyses for CDOM and turbidity are plotted over the sonde data for consistency with other tests. During the San Francisco Bay cruise temperatures ranged from 17 to 22 ^{o}C and salinity ranged from 21.5 to 27 PSU.

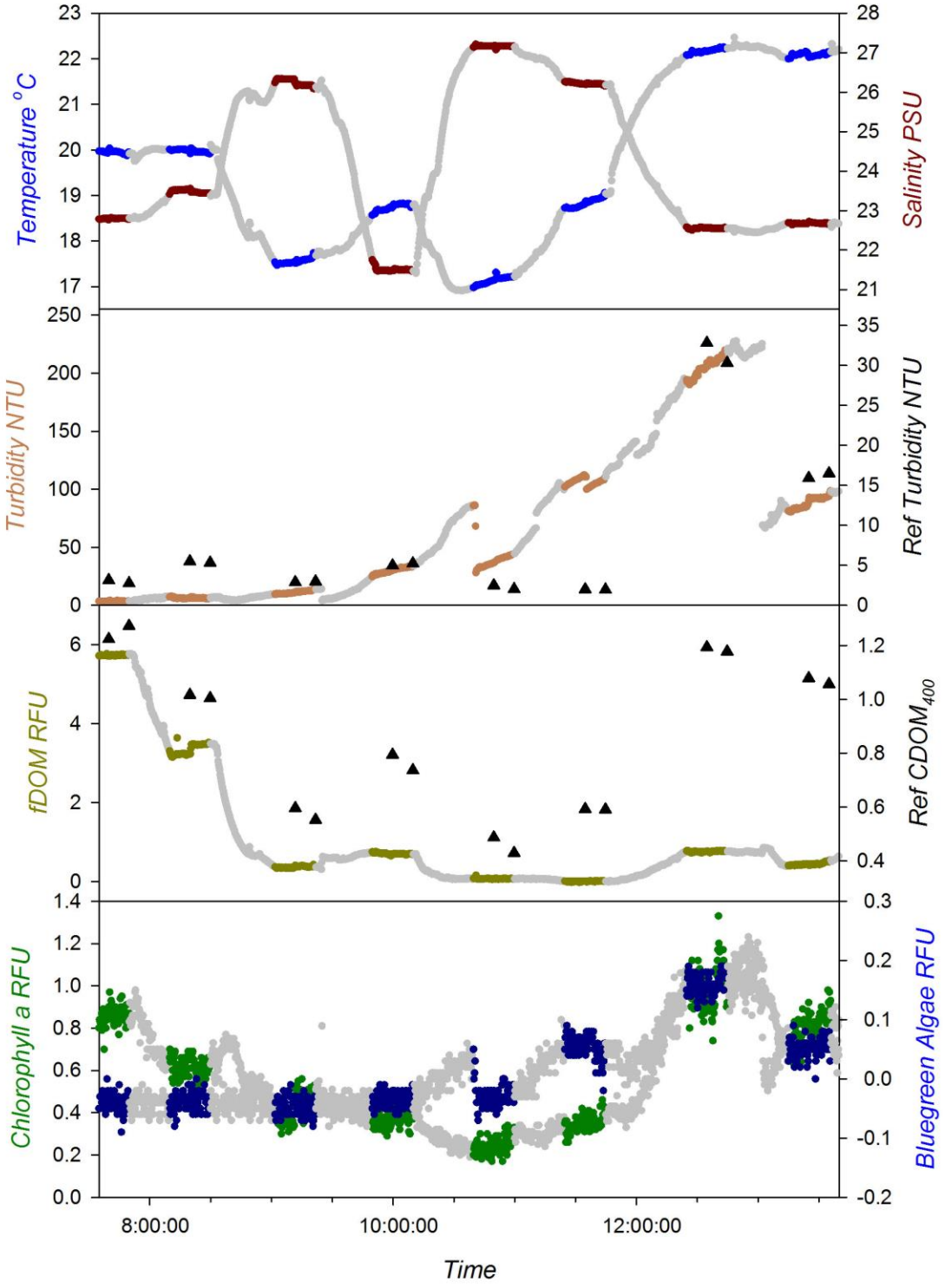


Figure 20. Time series of water conditions encountered during the surface mapping cruise in San Francisco Bay. *Top Panel:* Variation in temperature (blue) and salinity (red) at the depth of the sensors in the flow through tank, measured by an EXO 2 Sonde. *Second Panel:* Turbidity (brown) as measured by the EXO 2 and HACH 2100AN Turbidometer 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 $CDOM_{400}$ 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.

A time series of the CHL measurements from the PhytoFind and reference samples during the isolated exposures is shown figure 21. Extractable chlorophyll, a proxy for total phytoplankton biomass, ranged from 3.2 to 16.1 µg/L along the sampling transect with highest concentrations encountered in the southern end of South Bay (station 7). The PhytoFind CHL measurements ranged from 4.4 to 20.8 µg/L and were congruent with their corresponding reference samples. In contrast extractable phycocyanin, a proxy for cyanobacterial biomass, was low throughout the survey, ranging from 0 to 0.12 µg/L. The PhytoFind estimation of cyanobacteria (based on the % classification times total CHL) was below 0.5 µg/L for stations 1 – 6, about 3 µg/L for station 7 and 1 µg/L for station 8 (data not shown).

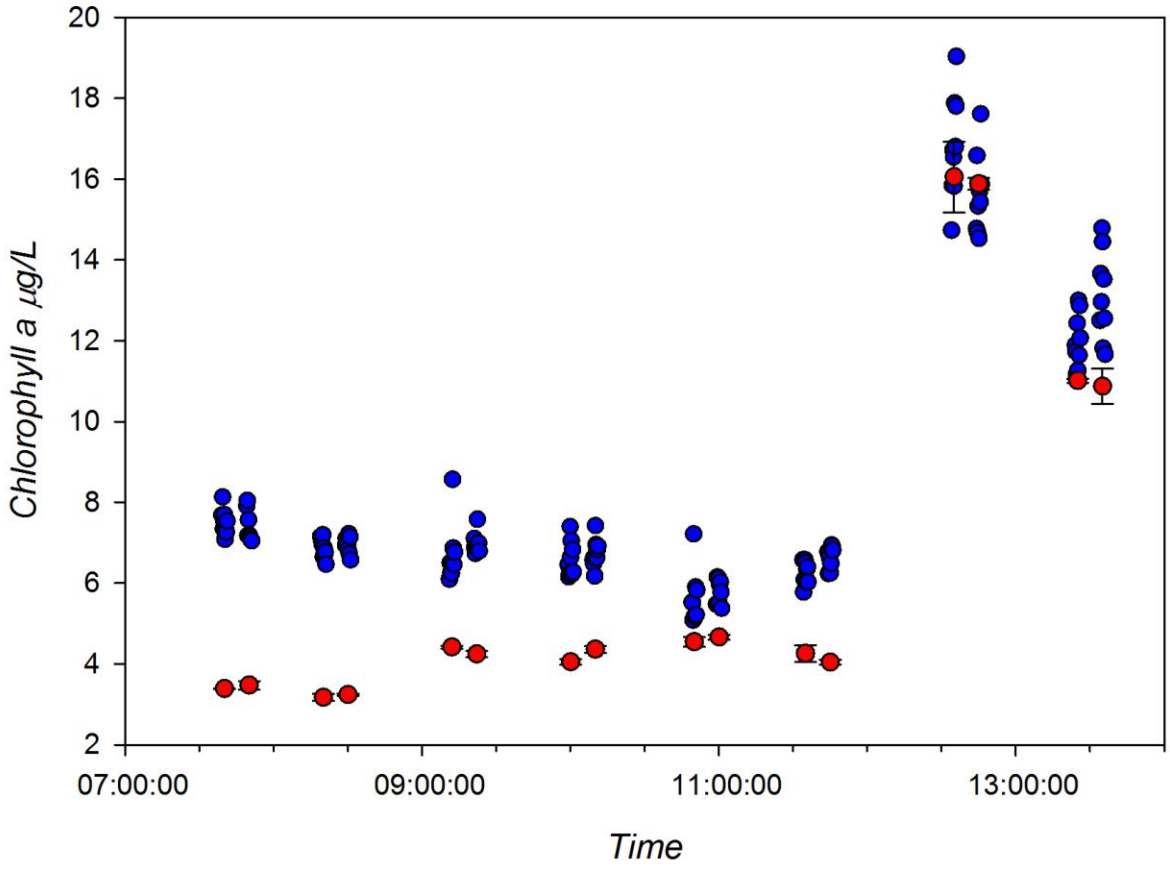


Figure 21. Plot of instrument (blue) and reference (red) measurements of chlorophyll a during San Francisco Bay surface mapping. Two reference measurements were made at each station, and the instrument data were averaged over two minutes bracketing the reference samples.

A linear regression of the PhytoFind chlorophyll measurements against the extracted chlorophyll (Figure 22) was highly significant ($p<0.001$) with an $R^2=0.95$ and a slope of 0.79.

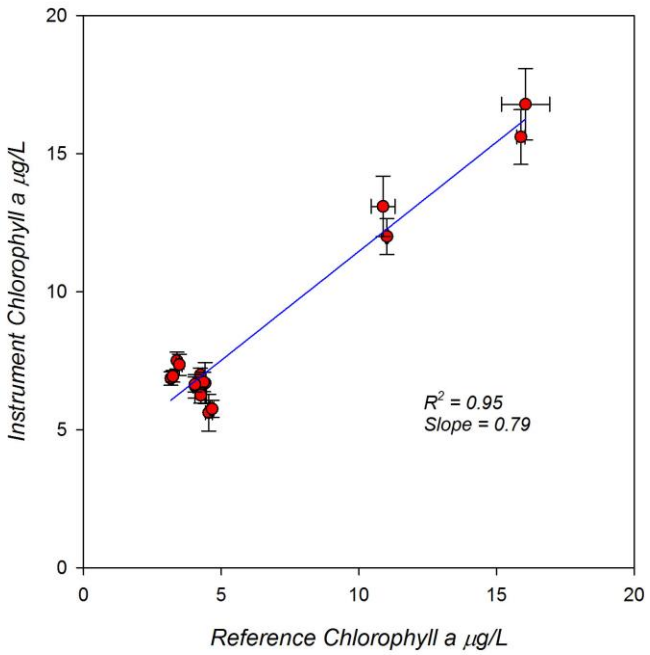


Figure 22. San Francisco Bay surface mapping response plot of the PhytoFind chlorophyll measurements compared to reference chlorophyll measured. The blue line represents the linear regression of the data.

Algal classification from the PhytoFind across the two measurement timepoints (T10 and T20) for each station isolation were very consistent for all eight sampling stations (Figure 23). The majority of the fluorescence signal was attributed to the Green and Brown algae. The contribution of fluorescence ascribed to Cyanobacteria or Mixed Algae ranged from a few percent at station 1 to a maximum of more than 40% at station 7. The proportion designated as Mixed Algae did not appear to be directly influenced by the amount of CDOM present (as measured from the reference samples). This suggests that the CDOM blanking procedure applied by the PhytoFind at the beginning of the survey helped eliminate the bias observed during the Lab tests with CDOM additions. In general, there was relatively good agreement between the PhytoFind and phytoplankton classifications generated with the onboard Imaging FlowCytobot (IFCB). One notable exception is that the ciliate portions of the counts were not assignable to a specific algal classification and specific cyanobacterial populations were not identified. The largest call-out of cyanobacteria by the PhytoFind occurred at the most optically complex water in the southern part of the bay where there was both high CDOM and high turbidity (Figure 23). Extracted PC at station 7 was barely measureable (0.03 µg/L) but higher than all other sites.

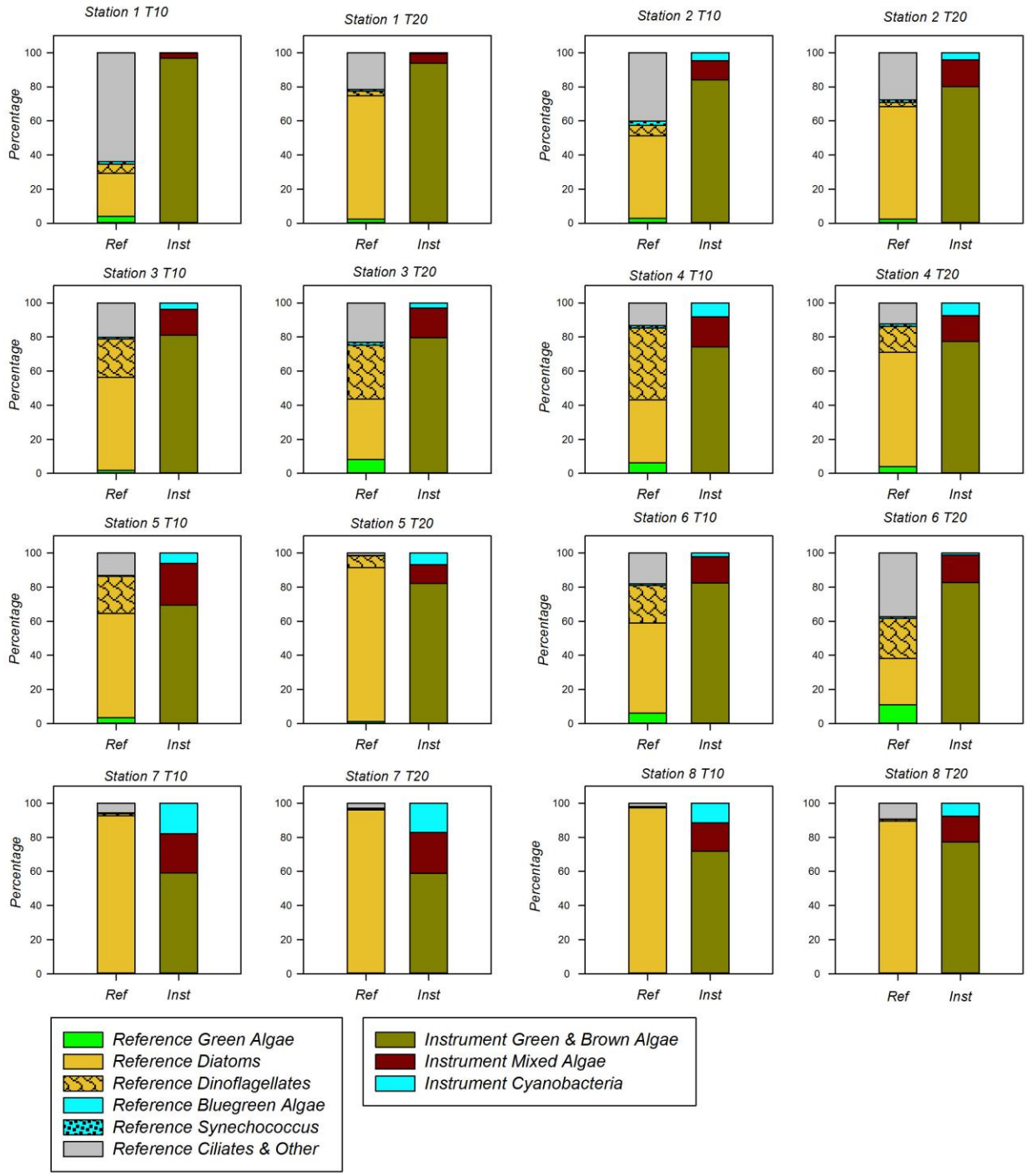


Figure 23. Reference cell counts as a percentage of biomass compared to instrument algae categories as a percentage of total chlorophyll a. Reference sample cell counts were estimated from UCSC Imaging Flow CytoBot.

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*, to assess instrument performance in near-shore to oceanic water conditions (*photo below*). *The R/V JH Martin* was equipped with a Headhunter – StingRay continuous flow pump which drew water via a through-hull port near the bow and 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, then along the 30 m isobath to assess near shore communities impacted by the combination of urban and agricultural watersheds feeding the coastal waters from Santa Cruz to Moss Landing Harbor.

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 24). Continuous measurements indicated that conditions in the tank during isolation periods were 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 PSU) and lower temperature waters (14 – 16.5 °C). Reference samples analyses for CDOM absorbance (0.03 to 0. 16) and turbidity (0.5 to 1.3 NTU) were also lower and less variable. Concentrations of extracted chlorophyll were similar in range (ca 5 – 15 µg/L) to SF Bay 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.

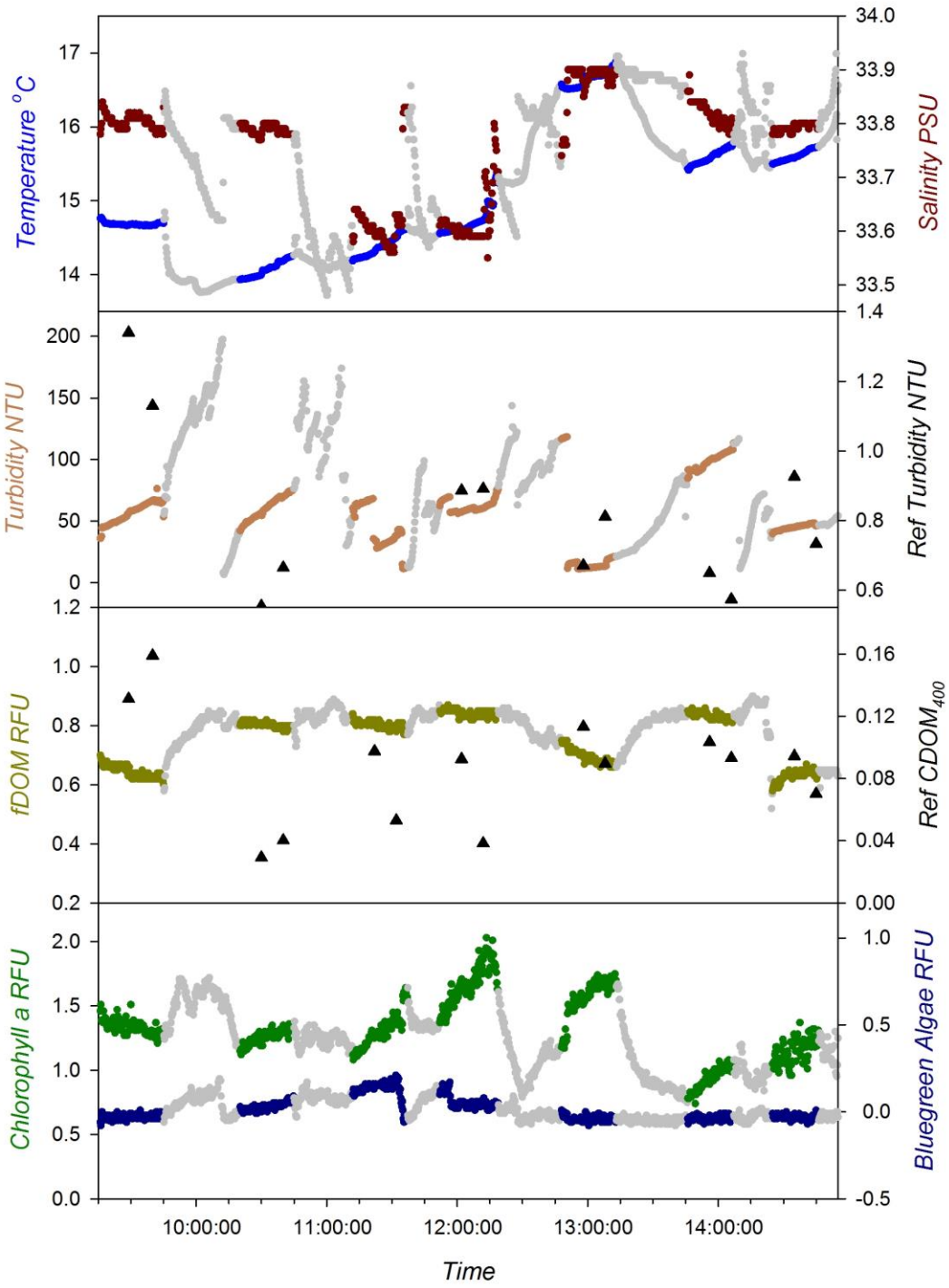


Figure 24. Water conditions encountered during the surface mapping in Monterey Bay. *Top Panel:* Variation in temperature (blue) and conductivity (red) at the depth of the sensors in the flow through tank, measured by an EXO 2 Sonde. *Second Panel:* Variation of turbidity (brown) as measured by the EXO 2 and discrete samples (black triangles) taken from the tank during reference sampling and measured on a HACH 2100AN Turbidimeter. Transients observed between reference sample periods in the EXO 2 time series for T, S and fDOM, reflect periods of partial draining of the exposure tank to promote enhanced water exchange for the next reference sample. *Third Panel:* fDOM (olive) as measured by the EXO 2, and $CDOM_{400}$ measured in discrete samples on an Agilent 8453 spectrometer (black triangles). *Bottom Panel:* Time series of dissolved chlorophyll a (green) and bluegreen algae (blue) as measured by the EXO 2 Sonde.

During the survey the PhytoFind produced 1273 readings (8 values were considered outliers) for a successful data return of 99%. CHL estimations from the PhytoFind ranged from 4.9 to 17.3 µg/L over the entire survey (Figure 25). The range in extracted phycocyanin from reference samples was 0.1 to 0.3 µg/L and correspondingly the PhytoFind called out a maximum of 1% contribution of cyanobacteria. Phytoplankton community compositions encountered along the survey transect are described below.

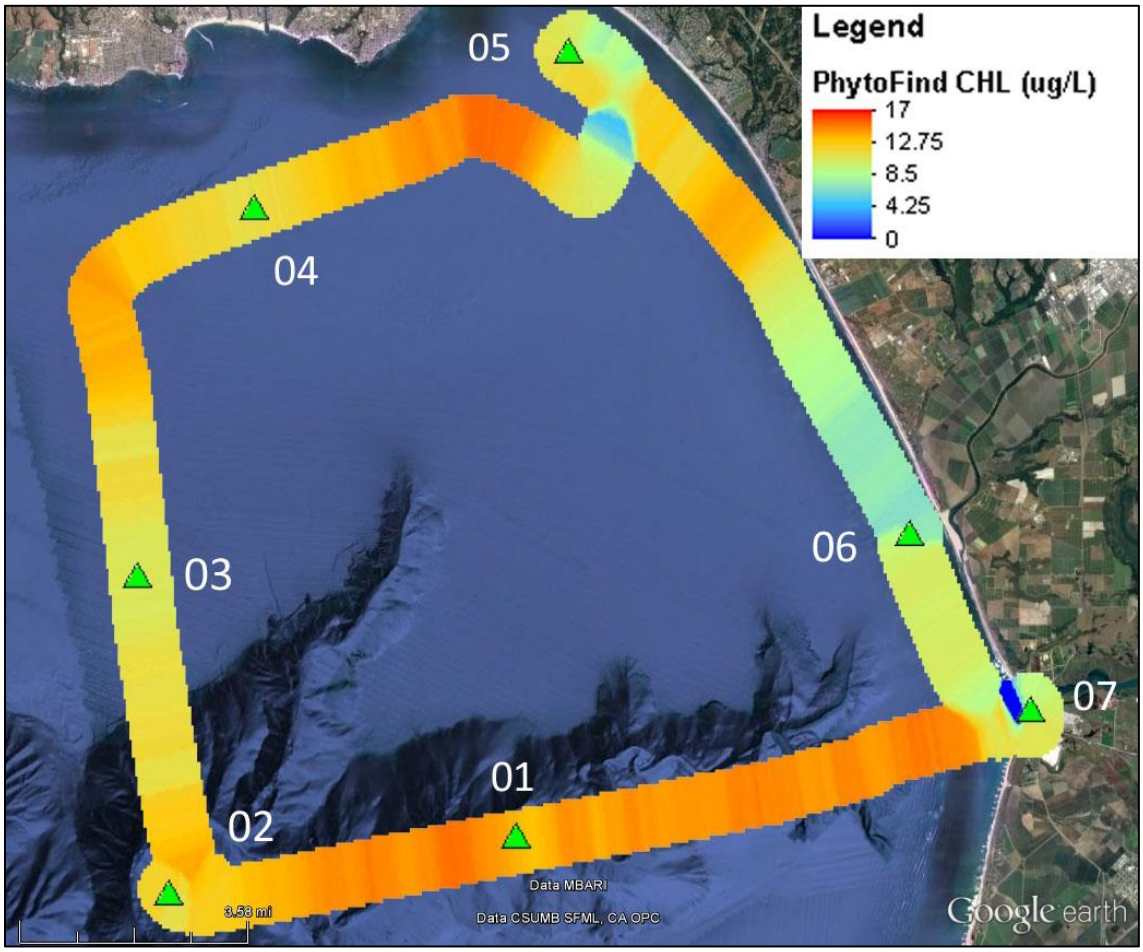


Figure 25. PhytoFind chlorophyll data contours during the continuous underway surface mapping cruise in Monterey Bay onbaord the *R/V JH Martin*. Triangles denote stations where the flow-through tank was isolated and comparative reference samples analyzed.

A time series of the PhytoFind CHL measurements are plotted against the corresponding reference measurements for the isolated exposure stations (Figure 26). For the isolated exposures, PhytoFind CHL measurements ranged from 8 to 16 µg/L compared to a range of 4.8 to 14.7 µg/L for the reference data. There was, however, significant variability in both the PhytoFind and reference sample grabs within the tank during the isolation period which limits the ability to accurately assess the response. To that point, the linear regression for instrument versus reference CHL estimation was not significant ($p=0.25$) with an $R^2=0.11$ and a slope of 0.21 (Figure 27).

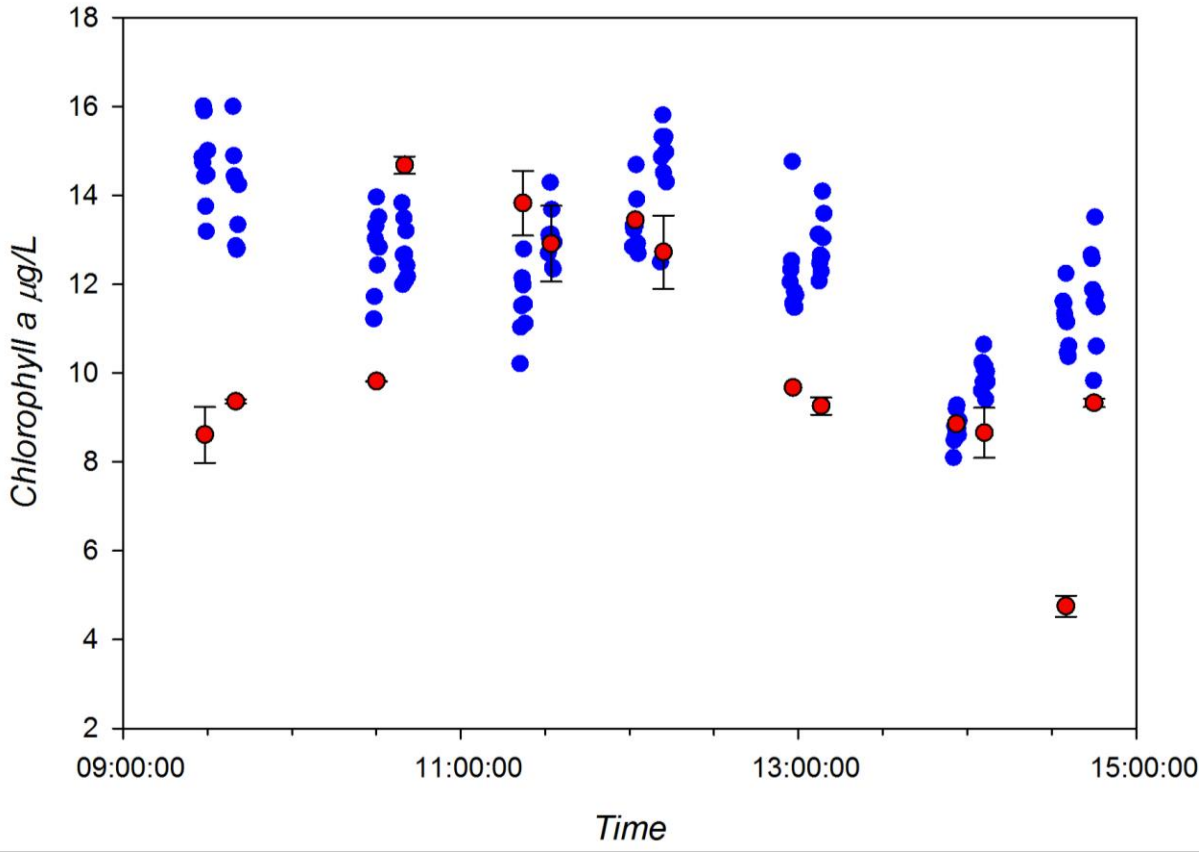


Figure 26. Plot of instrument (blue) and reference (red) measurements of chlorophyll a during Monterey Bay surface mapping. Two reference measurements were made at each station level while instrument measurements were averaged over 2 minutes bracketing the reference sample.

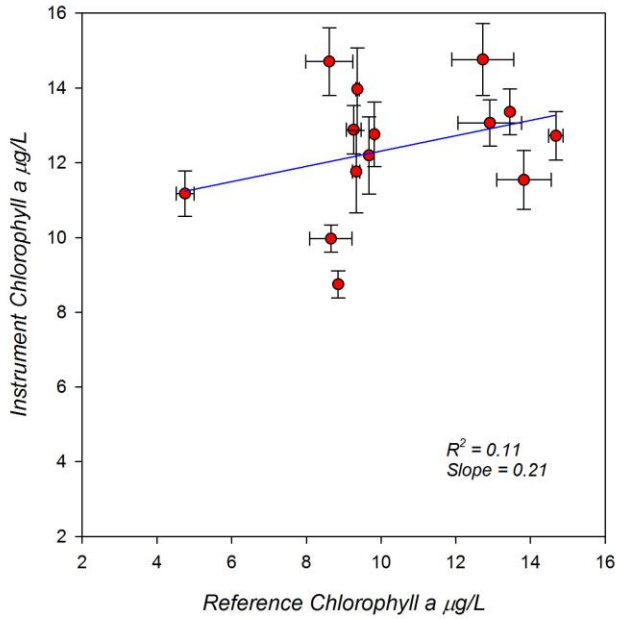


Figure 27. Monterey Bay surface mapping response plot of the PhytoFind chlorophyll a measurements compared to reference chlorophyll a measured in µg/L. The blue line represents the linear regression of the data.

Algal classification from the PhytoFind across the two timepoints (T10 and T20) for each station isolation were generally consistent for all seven sampling stations (Figure 28). The vast majority of the fluorescence signal for the PhytoFind was attributed to the Green-Brown category with only a small contribution (2-15%) attributed to the Mixed algal category. The highest contribution of Mixed Algae was noted for station 2 in the deeper offshore waters. While extracted PE was barely detectable at this site, it was the site of highest extracted PC but at a value of only 0.26 µg/L. Microscopic analysis of reference samples generally found cells within the Green, Diatom, and Dinoflagellate groups which would all map onto the PhytoFind Green-Brown classification. Microscopy also noted a fairly high abundance of ciliates and nanoplankton which could not be assigned to a specific algal group, but could be contributing to a small amount of Mixed algae signatures for the PhytoFind. As noted for the SF Bay mapping, the proportion designated as Mixed did not appear to be influenced by the amount of CDOM present (as measured from the reference samples). However, the amount and range of measured CDOM was low.

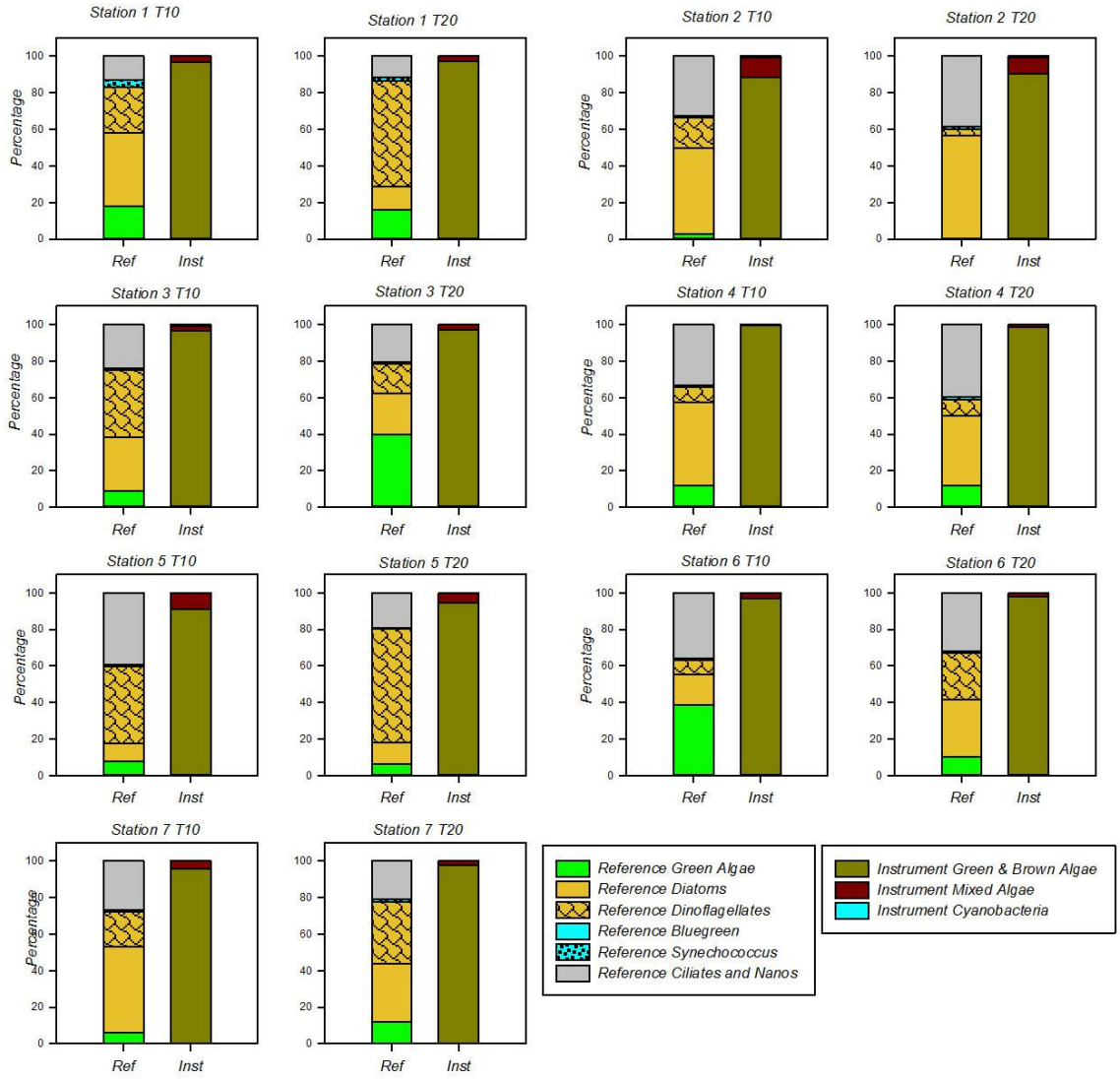
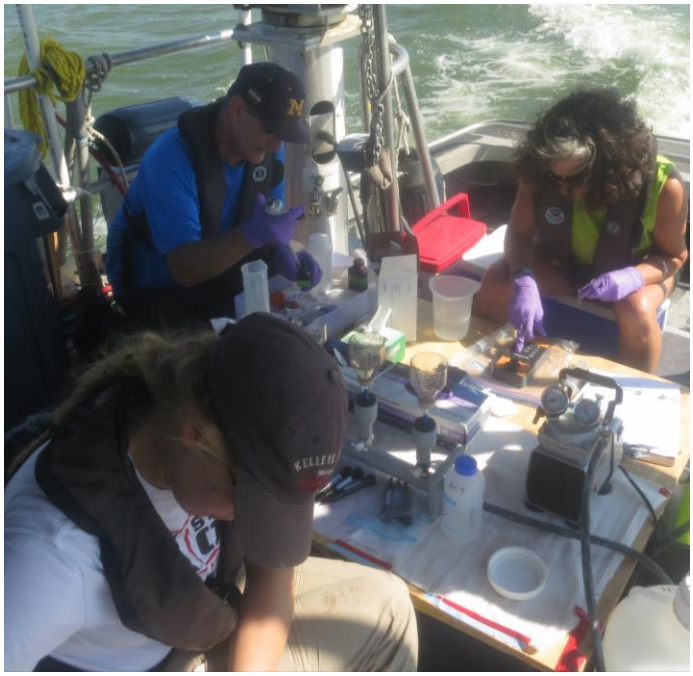


Figure 28. Reference cell counts as a percentage of biomass compared to instrument algae categories as a percentage of total chlorophyll a.

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 below*). The survey covered a 75 km range, including sites from near the mouth of the Maumee River out to open waters 20 km offshore. The survey occurred during an intense cyanobacterial bloom dominated by *Microcystis*. During the underway mapping cruise, seven stations were selected to make 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.



A YSI EXO2 sonde in the tank provided continuous monitoring results during the underway and isolated time periods with measurements taken every 15 seconds (Figure 29). Continuous measurements indicated that conditions in the tank during isolation were more variable at high bloom stations, likely reflecting 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 continuos CHL and BGA readings from the sonde showed significant spikes in cyanobacterial abudance as the survey transited nearshore, especially outside of Maumee Bay.

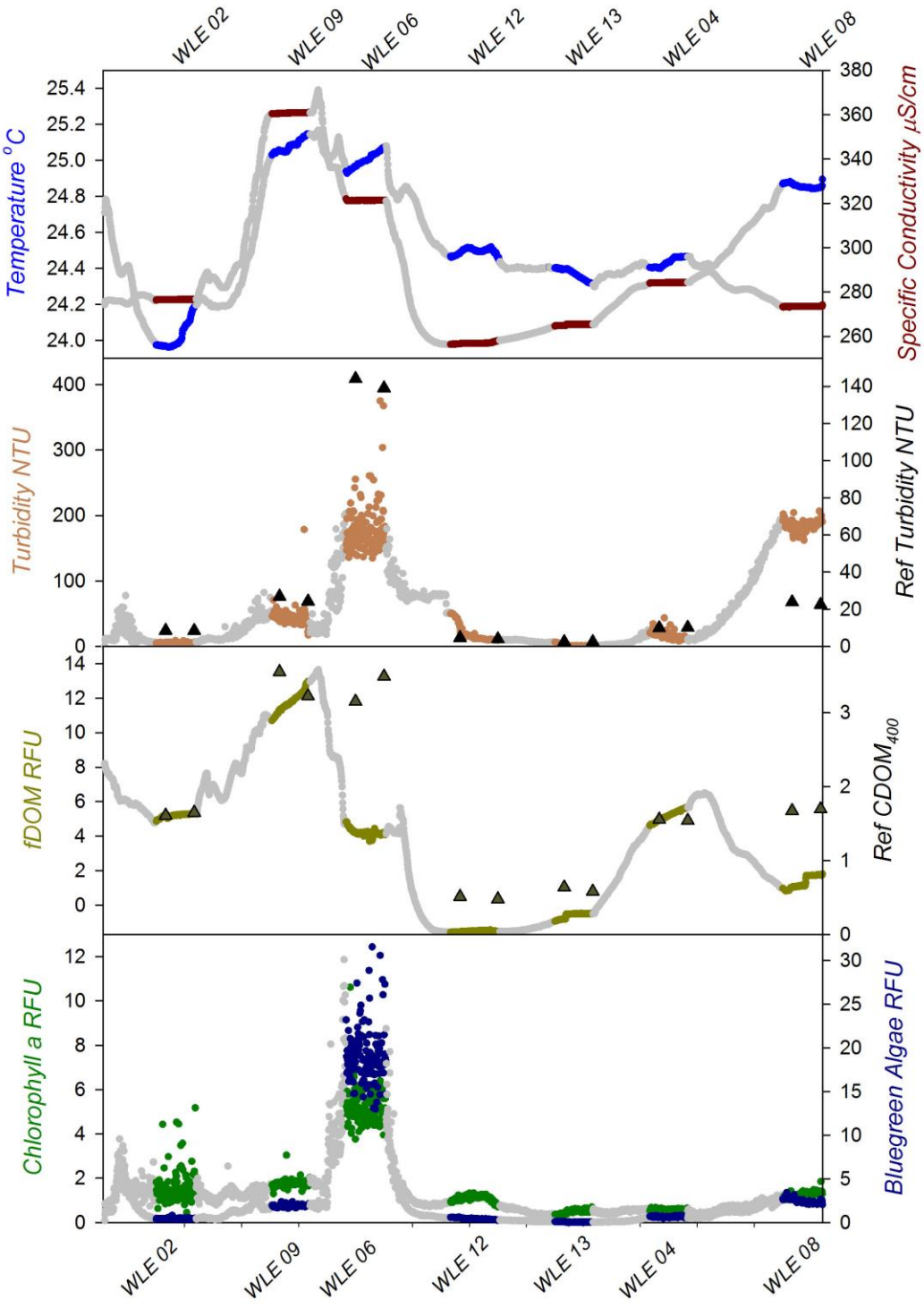


Figure 29. Water conditions encountered during the surface mapping in Western Lake Erie. *Top Panel:* Variation in temperature (blue) and conductivity (red) at the depth of the sensors in the flow through tank, measured by an EXO 2 Sonde. *Second Panel:* Variation of turbidity (brown) as measured by the EXO 2 and discrete samples (black triangles) taken from the tank during reference sampling and measured on a HACH 2100AN Turbidometer. *Third Panel:* fDOM (olive) as measured by the EXO 2, and $CDOM_{400}$ measured in discrete samples on an Agilent 8453 spectrometer (black triangles). *Bottom Panel:* Time series of dissolved chlorophyll a (green) and bluegreen algae (blue) as measured by the EXO 2 Sonde.

During the survey, the PhytoFind produced 2076 readings, of which 4 values were considered outliers for a successful data return of 99.9% (Figure 30). CHL estimations by the PhytoFind ranged from 5.2 to 215 µg/L over the entire survey compared to a range of 11 to 833 µg/L for extracted chlorophyll samples. The range in extracted phycocyanin from reference samples was 0.9 to 705 µg/L (data not shown) and the percent contribution of cyanobacteria called out by the PhytoFind ranged from 31 to 90%. Algal classifications during the survey are described below.

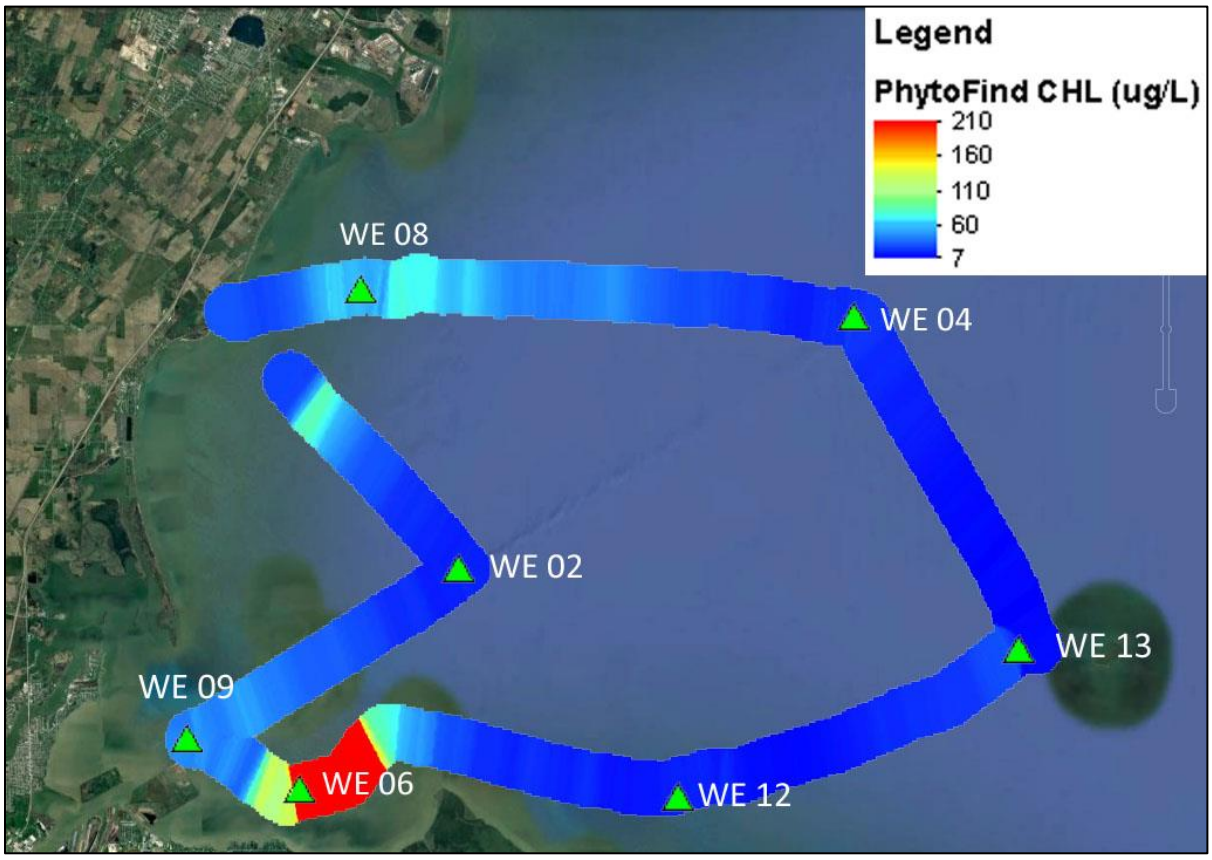


Figure 30. PhytoFind continuous underway chlorophyll data contours during the surface mapping cruise in Western Lake Erie. Triangles denote stations where the flow-through tank was isolated and comparative reference samples analyzed.

A time series of the PhytoFind and corresponding reference sample chlorophyll measurements for the isolated exposures is plotted in figure 31. CHL measurements for the PhytoFind during the isolation periods ranged from 5.17 to 215 µg/L and were consistently lower than the observed reference sample concentrations which averaged from 11 to 793 µg/L over the isolation timepoints. Pigment concentrations at station WE06 were clearly out of range for any meaningful in situ fluorescence measurement.

A one-to-one cross plot of the PhytoFind versus reference sample measurements, with data from WE06 omitted, is shown in figure 32. The linear regression was significant ($p<0.001$) with an $R^2=0.93$, and a slope of 0.52. The much lower instrument response per unit of extracted chlorophyll was not completely unexpected since it is well known that *Microcystis* colonies, like those present during this bloom, have very low fluorescence responses.

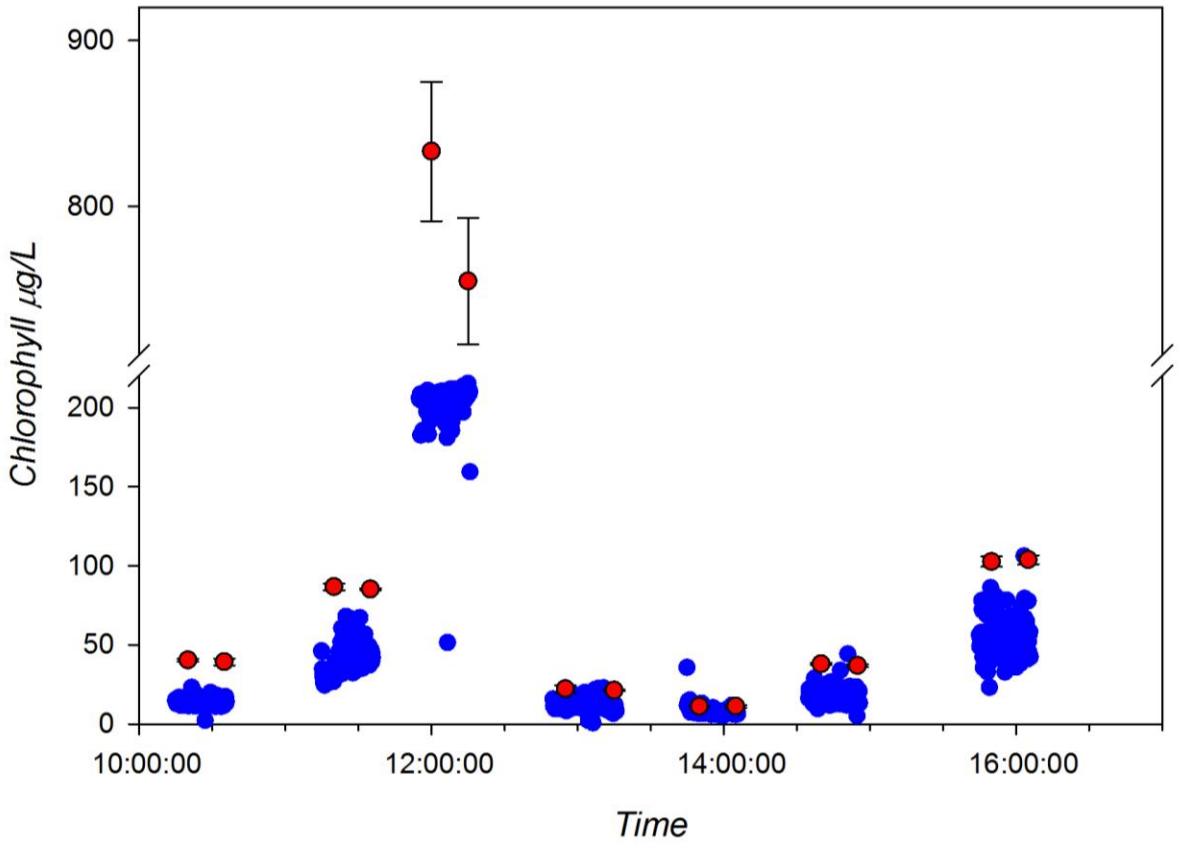


Figure 31. Time series plot of the PhytoFind chlorophyll (blue) and reference (red) during surface mapping deployment on Lake Erie. Two reference measurements were made at each station, and instrument data were averaged over 2 minutes bracketing the reference sample time.

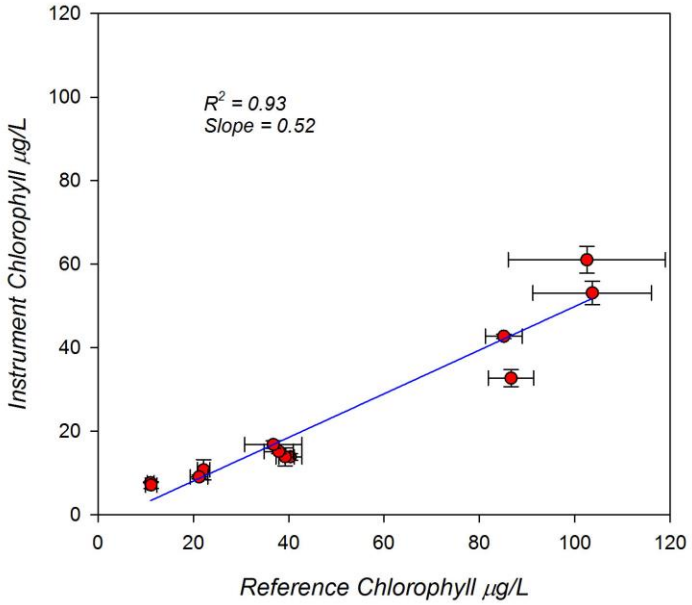


Figure 32. Lake Erie surface mapping response plot for the PhytoFind chlorophyll measurements compared to reference chlorophyll a. The blue line represents the linear regression of the data.

Algal classification from the PhytoFind was consistent across the two timepoints (T5 and T20) for each isolated sample for all seven stations despite the variability noted from the continuous sonde data (Figure 33). A significant contribution from Cyanobacteria was measured by the PhytoFind at all stations, but with the greatest proportions found at stations WLE 06 and WLE 08 which generally agreed with microscopy and extracted pigment results. However, the percent contribution from Cyanobacteria at the other stations was higher from the PhytoFind than found in cell counts. Cell counts had a significantly higher abundance of diatoms than was classified by the PhytoFind. Cell counts also had a low percentage of Cryptophytes, whereas the PhytoFind attributed between 10 to 40 % to the comparable Mixed algae category. The contribution of Mixed algae was inversely proportional to the amount of CDOM, which may reflect the use of a single initial sample to blank for CDOM effects. Overall, it appears that the Mixed Algae classification was a misrepresentation of diatoms.

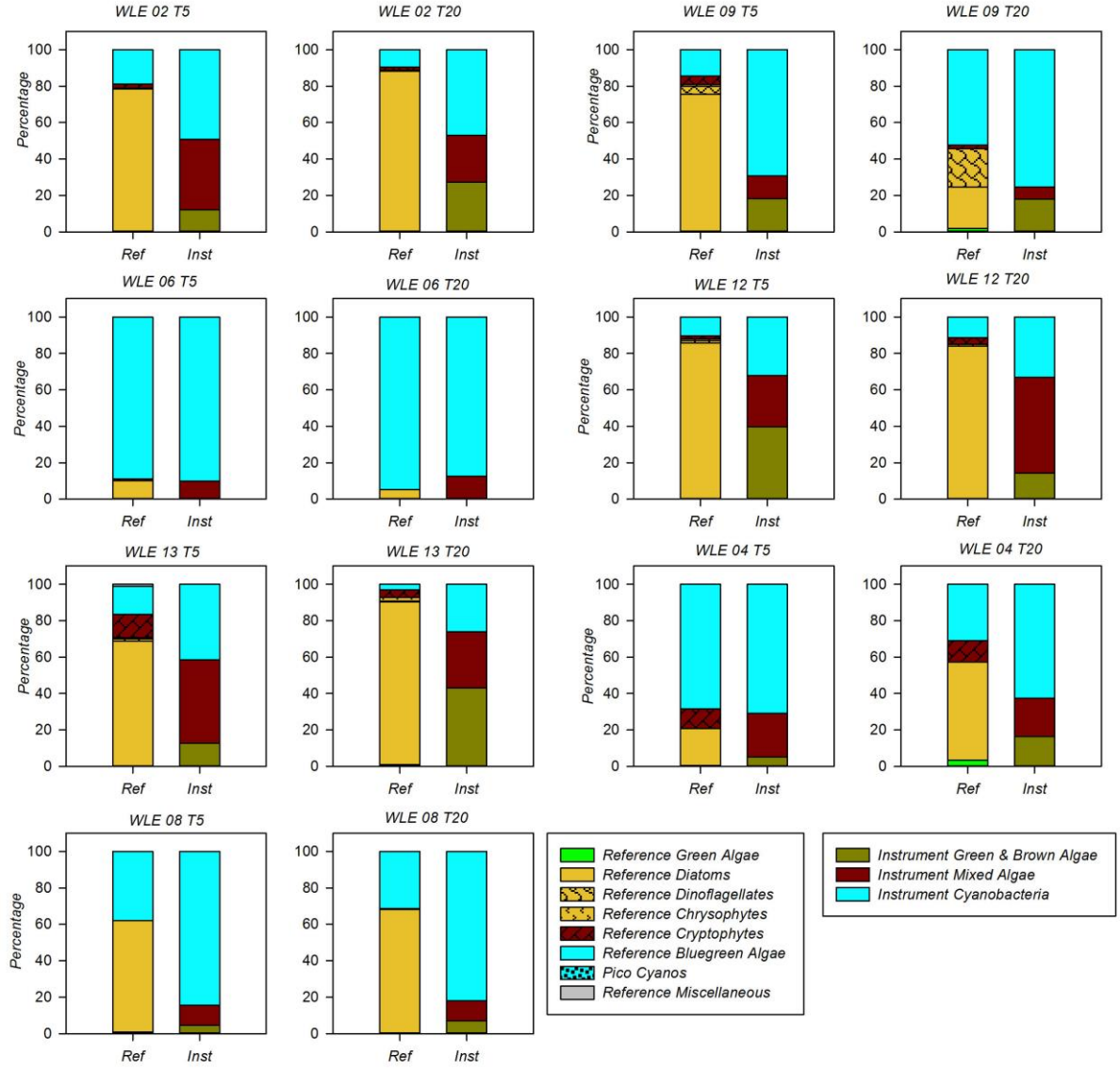


Figure 33. Reference cell counts as a percentage of biomass compared to instrument algae categories as a percentage of total chlorophyll a.

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 34). 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 upriver 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 34. 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).

During this moored deployment the PhytoFind collected 220 accepted observations with only 3 omitted outliers between July 25th and 30th (and two more isolated observations on 7/31) and then stopped due to a battery failure. The data return represented 36% of the planned test.

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 35. 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 – 29.4^{o}C and discharge covered a 5-fold range 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.

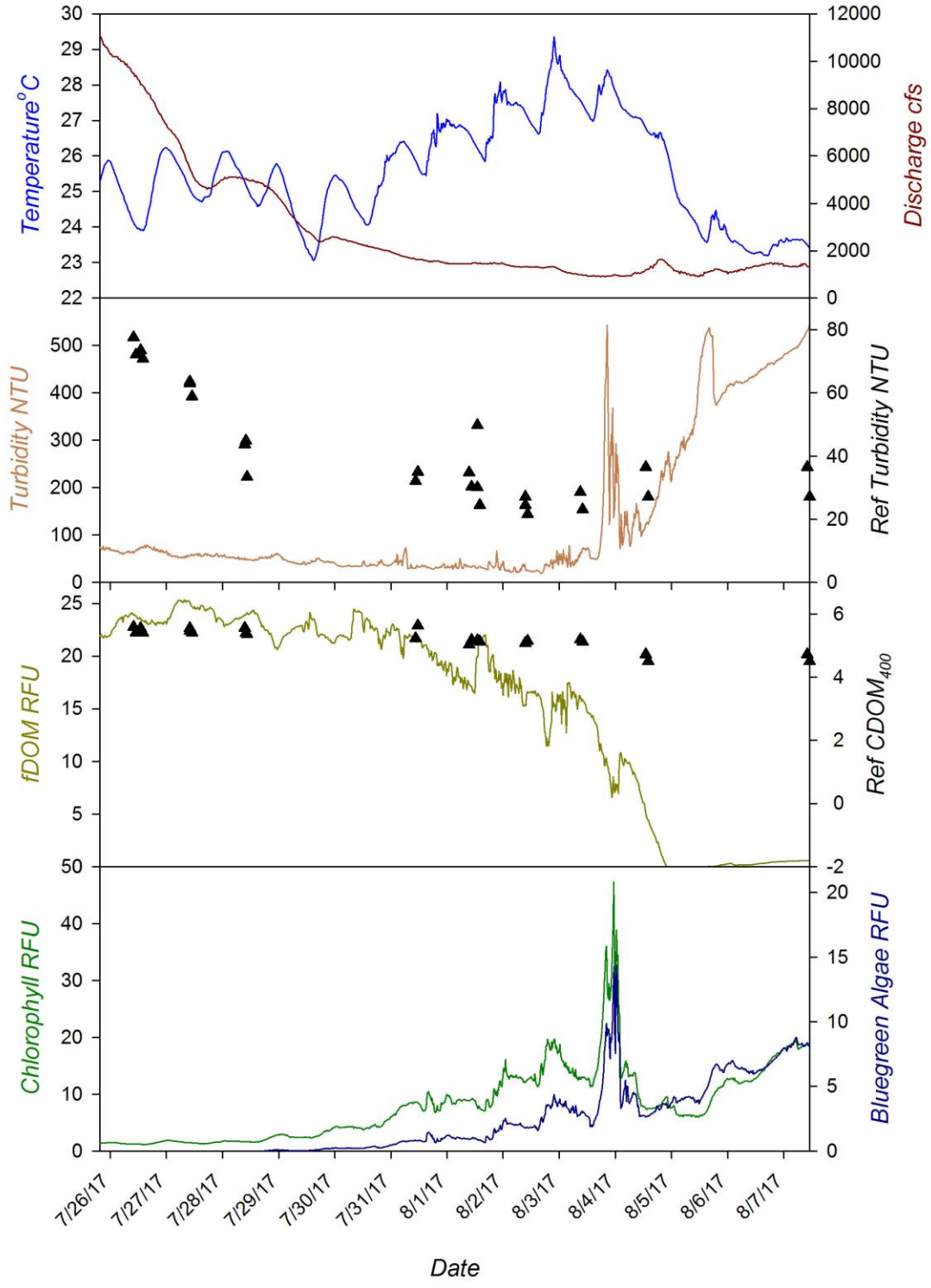


Figure 35. Environmental conditions encountered during the 13-day freshwater deployment in the Maumee River at Waterville, OH. *Top Panel:* Variation in temperature (blue) and Conductivity (red) at the depth of the sensors, measured by an EXO 2 Sonde. *Second and Third Panels:* Variation of turbidity (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 (green) and bluegreen algae (blue) as measured by the EXO 2 Sonde.

A time series of the PhytoFind CHL measurements against the corresponding reference sample CHL measurements for the Maumee River deployment is shown in figure 36. Chlorophyll measurements for the PhytoFind ranged from 9.98 to 69.3 µg/L during its period of operation. During that same period CHL from corresponding reference samples ranged from 9.5 to 69 µg/L, whereas the range for the total deployment reached 119 µg/L. The PhytoFind stopped recording data on 7/30 (02:30) due to a flooded battery, though it reported 2 final data points on 7/31 (15:00-15:30) that lined up with the nearby reference samples.

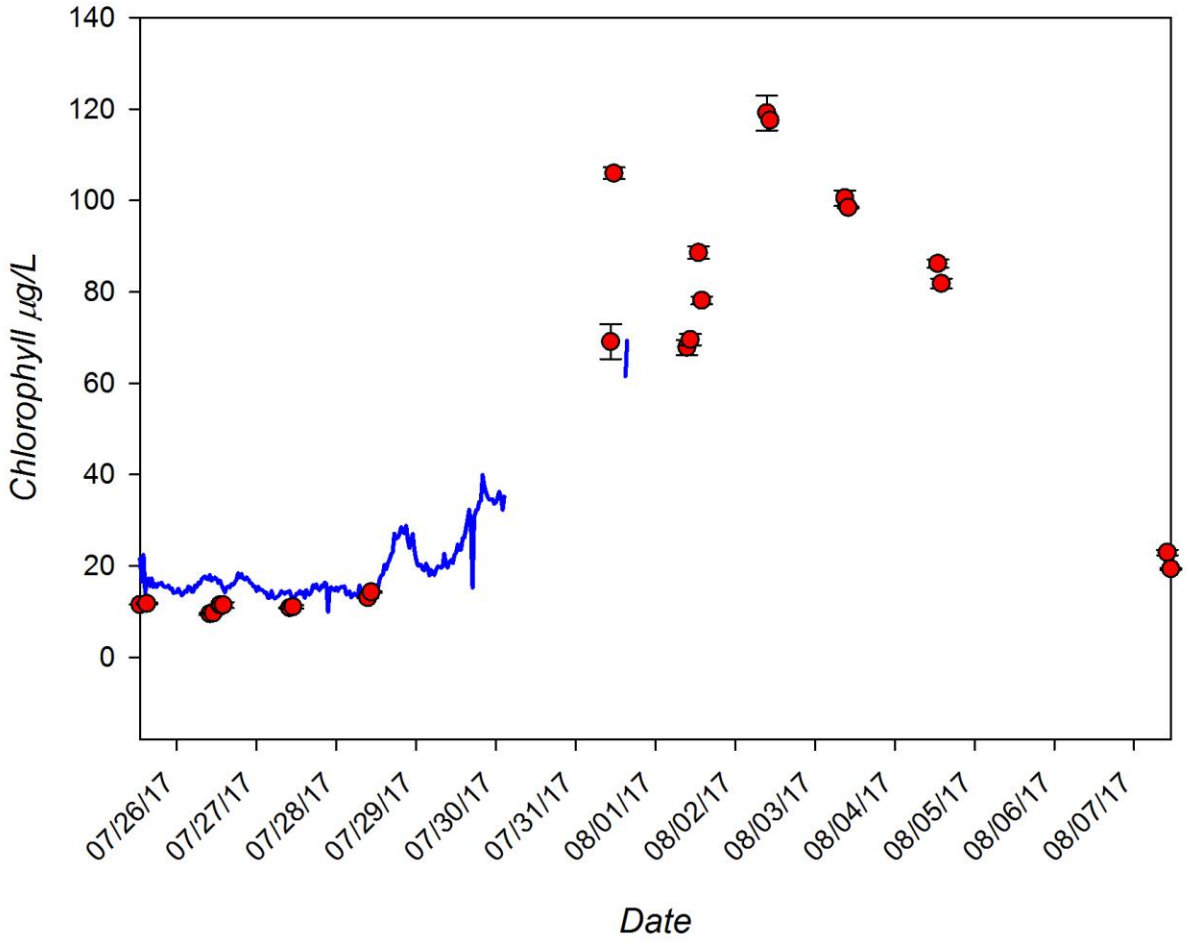


Figure 36. Time series plot of the PhytoFind measurements (blue) and reference measurements (red) of chlorophyll a during the freshwater deployment in the Maumee River at Waterville, OH. Water samples were typically collected 1 hour apart, with either two or four samples on a given day.

A one-to-one cross plot of the PhytoFind versus reference sample measurements is shown in figure 37. The limited data resulted in a linear regression that was not significant ($p<0.326$) with an $R^2=0.12$ and a slope of -0.61. The negative slope may indicate the ultimate failure was also affecting performance during its operating period, but this was also a very limited test range. It should be noted that the data from 7/31 is not included because the instrument observation was not at the exact time of the isolated reference sample.

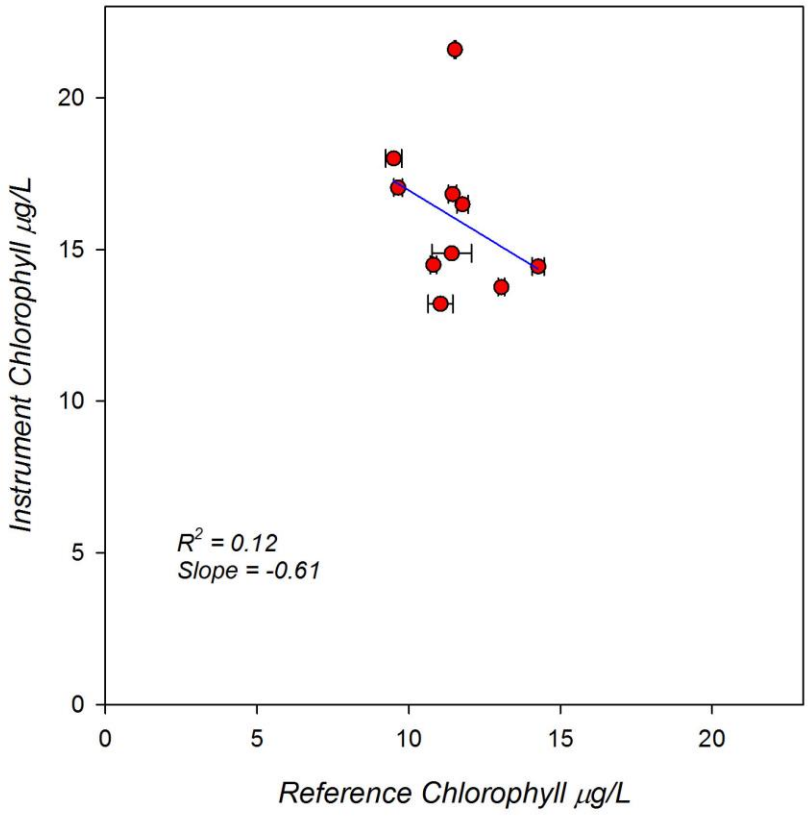


Figure 37. Cross plot of PhytoFind and reference sample measurements during the Maumee River field deployment. The data represent observations from 6 days of a 13-day deployment. The blue line represents the linear regression of the data.

A comparison of algal group classification from the PhytoFind and reference sample cell counts is given in figure 38. Data are available only for the first 5 days of the deployment due to early instrument failure. Unfortunately, these dates are all before a noteable increase in CHL and PC (as seen in the sonde data) and therefore provide a small range of composition and abundance. Two samples were taken each day, but were not true replicates since they were acquired one or two hours apart. The PhytoFind appeared to mis-assign the Greens, Diatoms, and Dinoflagellates into the Mixed Algae category in these waters. CDOM and turbidity were both singificantly higher in the river water than in the open Lake or coastal ocean, perhaps making the optically complex water more difficult to resolve fluorescently. Microscopic cell counts only found a measureable contribution to Cyanobacteria on July 27th when it contributed to approximately 10% of the biovolume, whereas the PhytoFind attributed approximately 20% of the fluorescent signal to Cyanobacteria for most samples. Extracted PC during these five days ranged between 0.1 to 0.6 µg/L, suggesting the cell counts more accurately characterized the phytoplankton community composition.

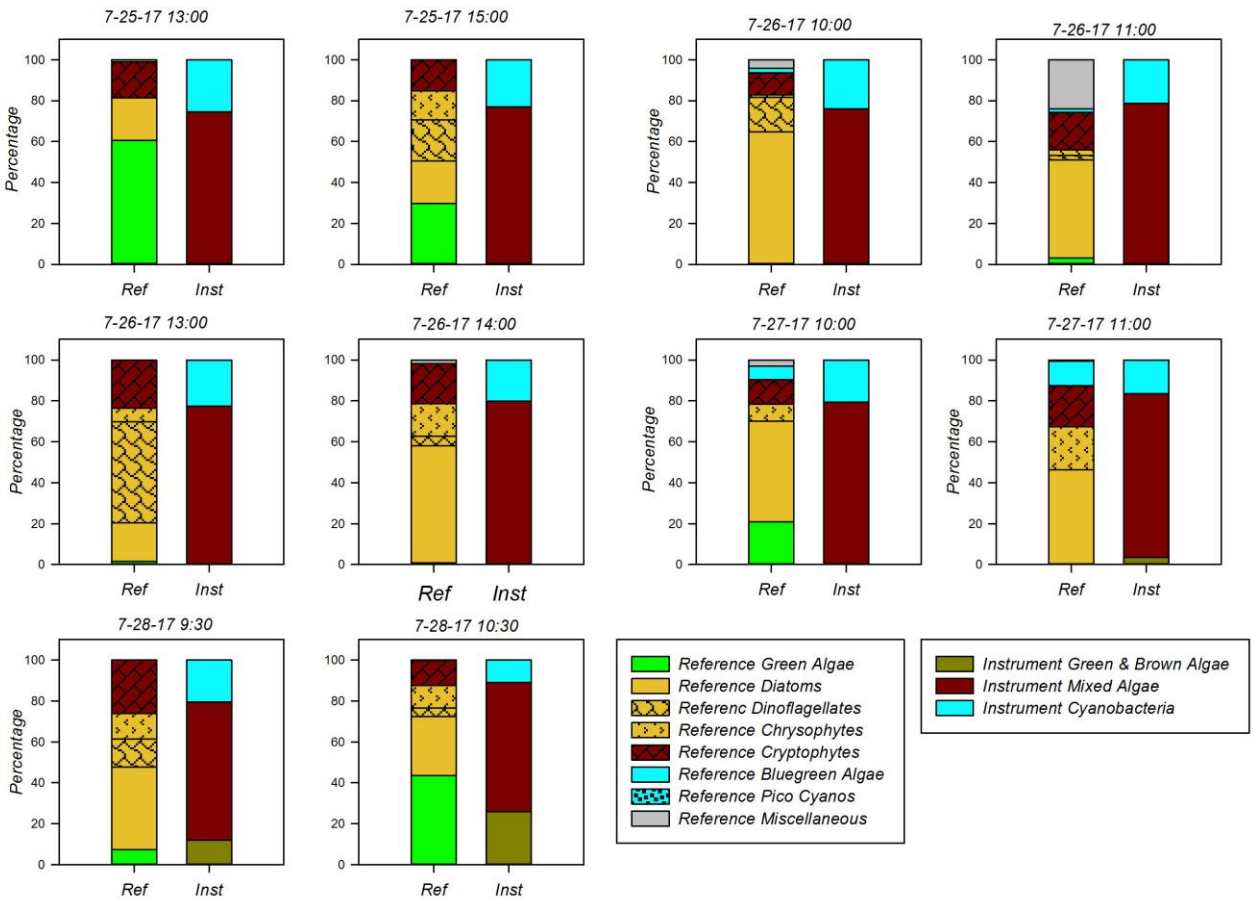


Figure 38. Reference cell counts as a percentage of biomass compared to instrument algae categories as a percentage of total chlorophyll-a estimated by the PhytoFind for the Maumee River field deployment test.

Figure 39. Instrument photographs prior to deployment (*top*) and upon retreival (*bottom*).

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 40). The site was brackish with an average water depth of 2.2 m at the test site.

Figure 40. Aerial view of CBL deployment site (*left*) and instrument rack deployed next to the dock (*right*).

The PhytoFind operated only for the first five days of this deployment and stopped on 9/11 at 14:00 due to another apparent battery failure. During its working period the PhytoFind collected 120 accepted observations (with 2 omitted outliers), however the data were much more variable than expected and may have indicated improper functioning. The data return represented 19% of the planned test. A brand new, fully charged battery was installed prior to the deployment but appeared to have leaked upon inspection after recovery.

Continuous monitoring of ambient conditions for temperature, salinity, turbidity, fDOM, chlorophyll and bluegreen algae measured by an EXO 2 sonde at 15 minute intervals are given in figure 41. 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.

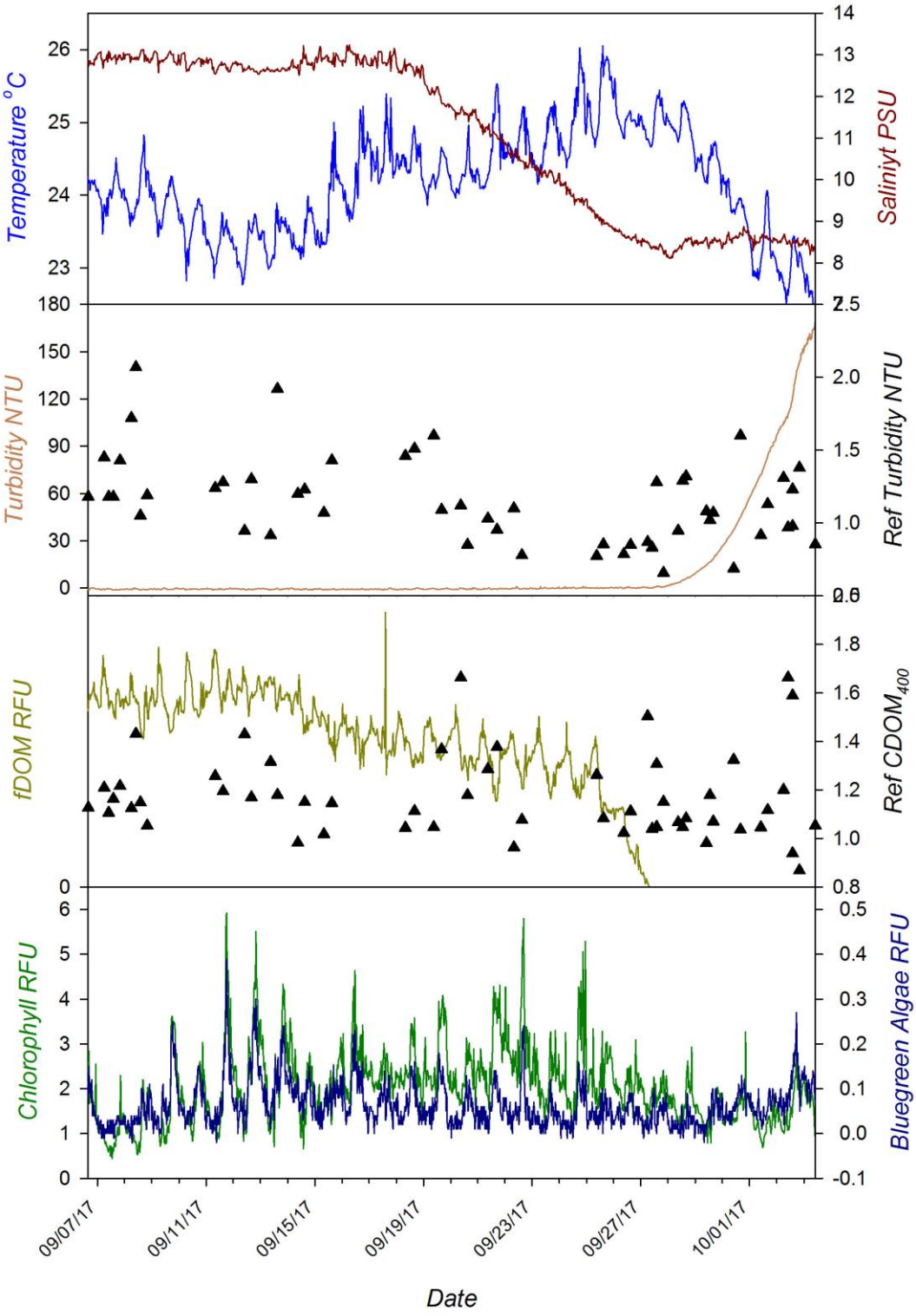


Figure 41. Environmental conditions encountered during the 28-day CBL moored deployment. *Top Panel:* Variation in temperature (green) and salinity (red) at depth of instrument sensor detected by an EXO2 sonde. *Second Panel*: Variation in turbidity (brown) as measured by the EXO 2 sonde and discrete samples measured on a HACH 2100AN (black triangles). *Third Panel:* fDOM (olive) as measured by the EXO 2 and $CDOM_{400}$ measured on an Agilent 8453 spectrometer (black triangles). *Bottom Panel:* Chlorophyll (green) and bluegreen algae (blue).

A time series of the PhytoFind measurements of chlorophyll a plotted against the corresponding reference measurements in figure 42. Chlorophyll measurements for the PhytoFind ranged from 3.6 to 28 µg/L compared to the range in reference samples of 8.2 to 15 µg/L during this same time period. For the entire test period, reference chlorophyll ranged from 7.4 to 21.7 µg/L. Phycocyanin concentrations from reference samples ranged from 0.6 to 5.5 µg/L and it should be noted that this was the only site where we also saw measureable levels of phycoerythrin in the reference samples, with concentrations ranging from 0 to 3.4 µg/L (data not plotted).

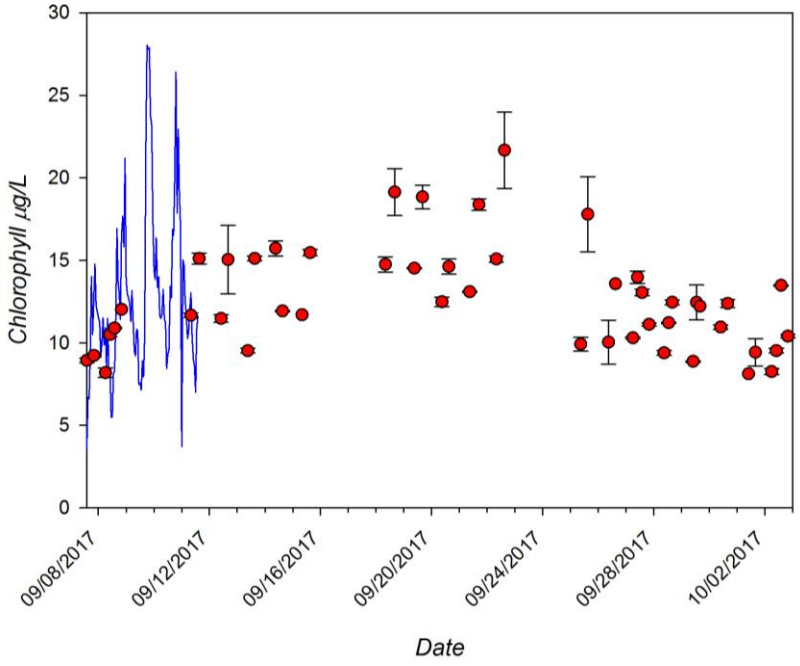


Figure 42. Time series plot of the PhytoFind (blue) and reference measurements (red) of chlorophyll a during the CBL moored deployment in Solomons, MD.

A one-to-one cross plot of the PhytoFind versus reference sample measurements of chlorophyll for the five days of data collection is shown in figure 43. The linear regression for CHL was significant (p=0.021) with an R^2=0.51 and a slope of 1.01.

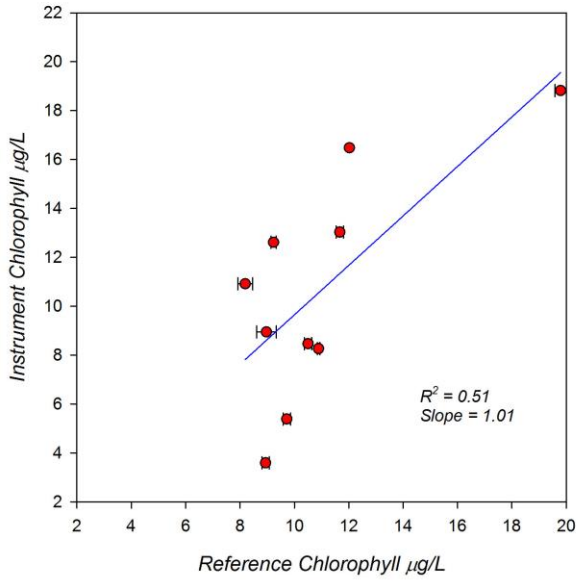


Figure 43. Cross plot of PhytoFind and reference sample measurements during the CBL field deployment. The data represent observations from 5 days of the 28-day deployment. The blue line represents the linear regression of the data.

A comparison of algal group classification from the PhytoFind and reference sample cell counts for the Chesapeake Bay deployment is given in figure 44. Data are available only for the first 6 days of the deployment due to early instrument failure. One comparative sample is available on September 6th and 11th, while four samples per day were collected on the 7th and 8th to capture finer-scale diurnal response. With the exception of the 9/7 14:00 sample, the PhytoFind classifications matched the microscopic biovolume classificatons reasonably well. For that sample, the PhytoFind mis-assigned Diatom and Dinoflagellates into the Mixed algae category. In general, the PhytoFind classified approximately 25% of the fluorescence contribution to the Mixed algae class, whereas counts suggested these contributions came from small *Synechococcus*. As noted above, this sampling site did have measureable amounts of extracted PE, which for these first five days ranged from 0.1 to 2.4 µg/L. However, the value was near the minimum on 9/7 14:00 so that abrupt change in the PhytoFind was not an accurate designation of community composition.

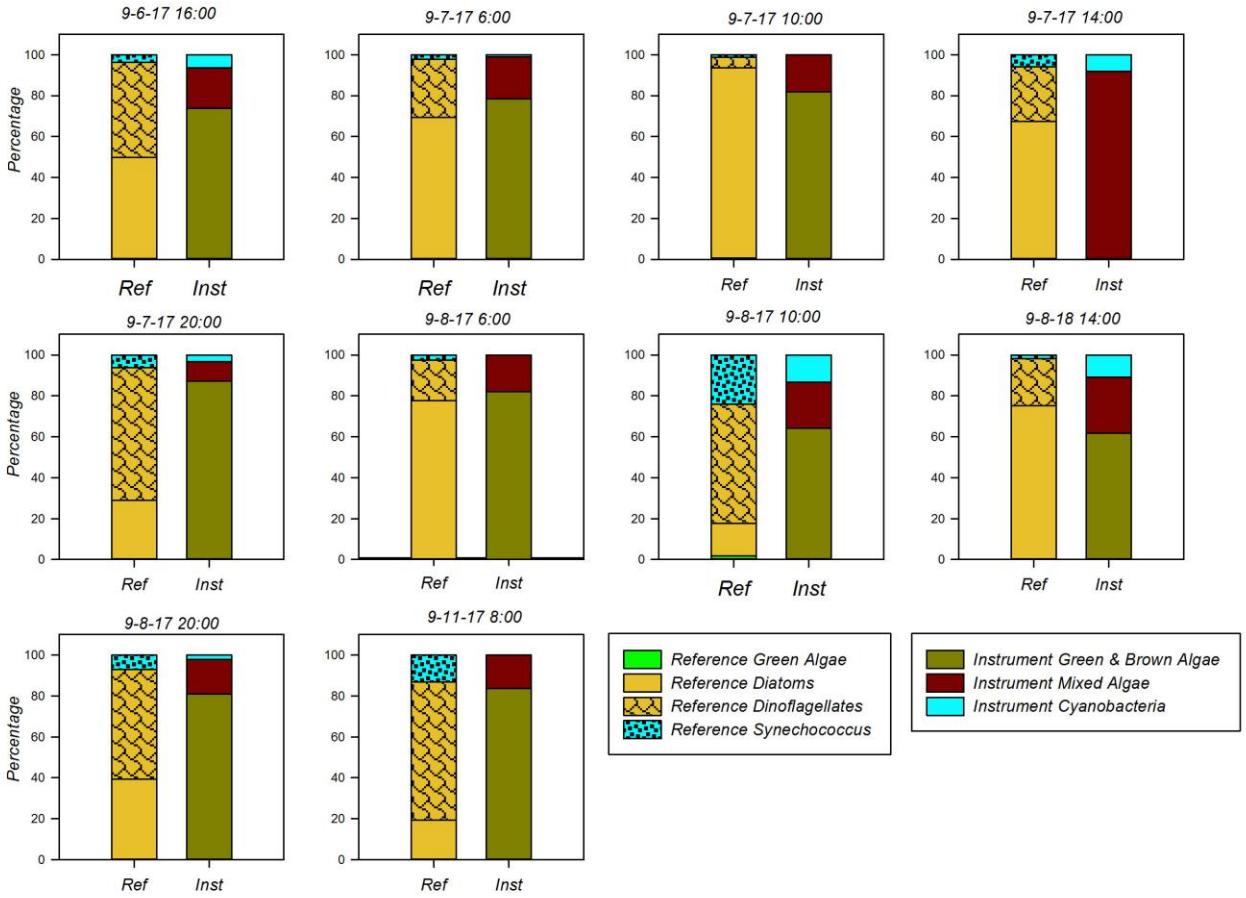


Figure 44. Reference cell counts as a percentage of biomass compared to instrument algae categories as a percentage of total chlorophyll estimated by the PhytoFind for the Chesapeake Bay field deployment test.

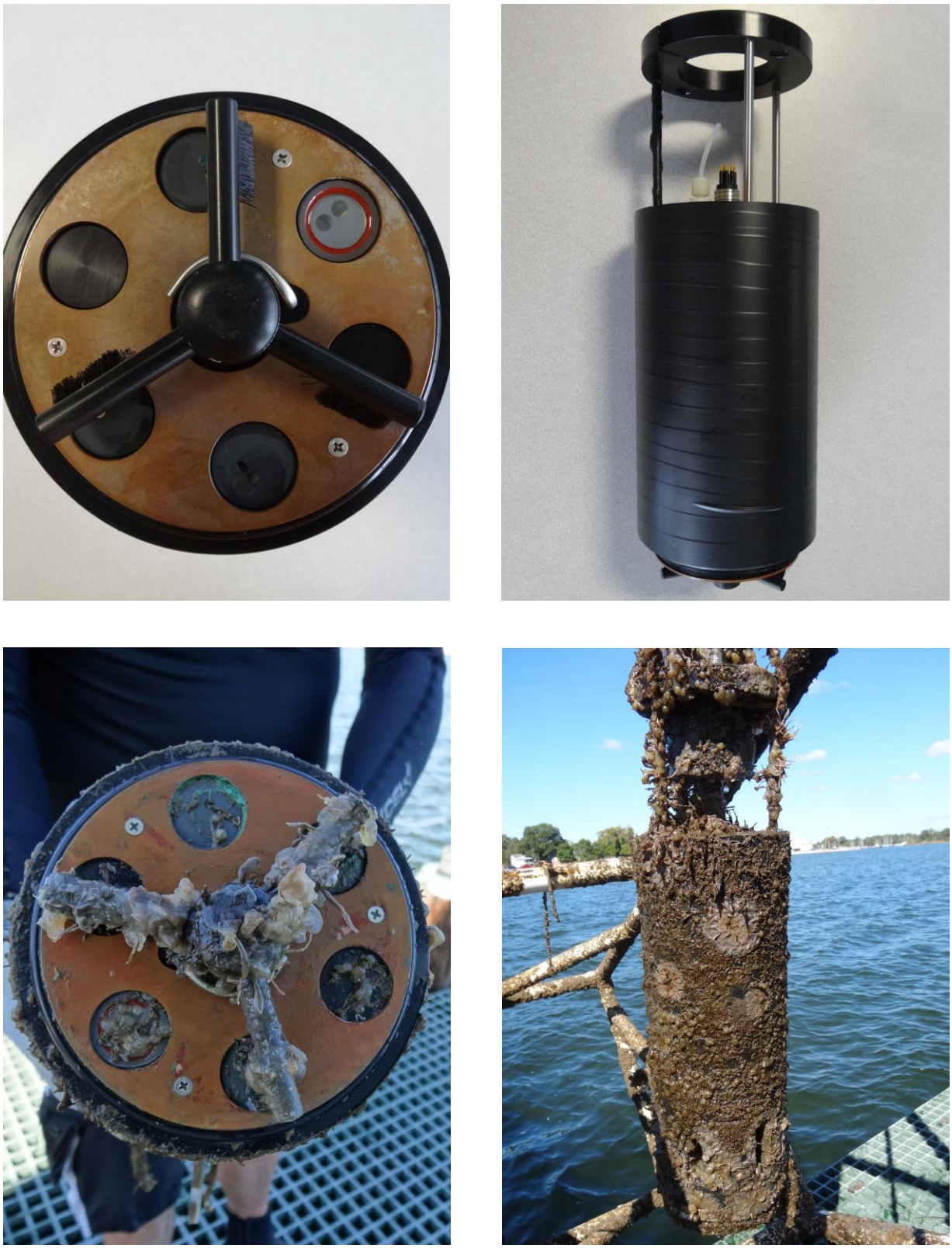


Figure 45. Instrument photographs prior to deployment (*top*) and upon retrieval (*bottom*).

GLOBAL RESPONSE

A cross plot of PhytoFind versus reference chlorophyll for all field tests were combined into a single plot (Figure 46). 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 0.21 for the Monterey Bay surface mapping to 1.01 for the CBL moored deployment. A single regression fit through all the data resulted in a linear regression ($p < 0.001$) with a slope of 0.25 and $R^2 = 0.97$. This regression is highly skewed by the high concentration in western Lake Erie. A regression without the Lake Erie results, which covered a much lower and narrower range, resulted in a regression ($p < 0.001$) with a slope of 0.79 and $R^2 = 0.54$.

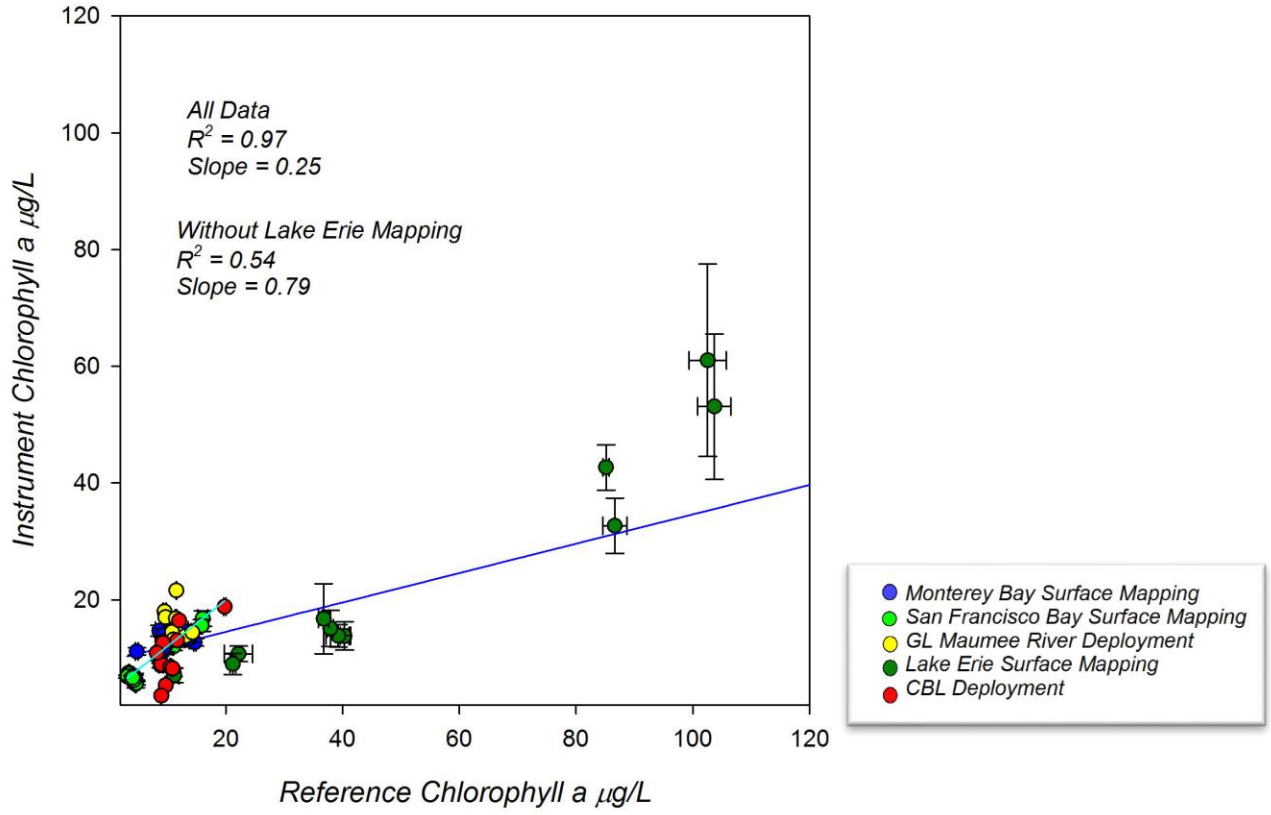


Figure 46. Global response plot for the PhytoFind CHL estimation compared to extracted chlorophyll for all five ACT field trials. The blue line represents the linear regression of all data and the cyan line represents the regression without Lake Erie data included.

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 competence of testing and calibration laboratories*.

Quality Control Sample Analysis

As part of the sample analysis quality control evaluation two field blank samples (Table 8) and two field duplicate samples (Tables 9-11) 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**

A comparison of microscopy results for field duplicate reference samples collected during the Maumee moored deployment test is shown in Table 11. Total biovolume differed by a factor of 2 at low cell abundance (GL10 and GL11), but with similar ratios of composition across species. At higher abundance (GL20 and GL21) total abundance agreed to within approximately 20% and relative species composition was consistent between the two replicates.

Table 11. Comparison of reference sample analysis for Field Duplicates from the Maumee River, OH mooring test.

Sample ID	GL10	GL11			GL20	GL21	
Sample Type	Ref	Field Dup			Ref	Field Dup	
	Biovolume (10^3 μm^3/mL)	Biovolume (10^3 μm^3/mL)	St Dev		Biovolume (10^3 μm^3/mL)	Biovolume (10^3 μm^3/mL)	St Dev
Greens	141	298	111		2081	1081	707
Diatoms	332	696	257		3794	5187	985
Bluegreens	1	181	127		0	5	na
Chrysophytes	1	229	161		150	343	137
Cryptophytes	165	0	na		731	1891	820
Dinoflagellates	0	0	na		875	1258	270
Miscellaneous	40	2	27		0	68	na
Syn/Picos	0.32	0.24	0.06		0.27	0.37	0.07
Total	**680**	**1406**	**513**		**7631**	**9833**	**1557**

A comparison of microscopy results for field duplicate reference samples collected during the Chesapeake Bay moored deployment test is shown in Table 12. For the first set of field replicates (CBL 41 and 42) total biovolume differed by a factor of 3 but the species composition ratios were fairly consistent. For the second set of field replicates (CBL 57 and 58) total biovolume differed by only 15% and the species composition ratios were very consistent.

Table 12. Comparison of reference sample analysis for Field Duplicates from the Chesapeake Bay, MD mooring test.

Sample ID	CBL41	CBL42			CBL57	CBL58	
Sample Type	Ref	Field Dup			Ref	Field Dup	
	Biovolume (10^3 m^3/mL)	Biovolume (10^3 m^3/mL)	St Dev		Biovolume (10^3 m^3/mL)	Biovolume (10^3 m^3/mL)	St Dev
Diatoms	656	1596	665		1713	1676	26.2
Dinoflagellates	232	1323	772		122	475	249
Euglenoids	21.4	21.4	0		0	0	0
Syn/Picos	54.0	79.8	18.3		57.8	54.3	2.5
Total	**964**	**3021**	**1455**		**1894**	**2206**	**220**

Technical System Audits

An effective assessment program is an integral part of a quality system. The ACT Quality Assurance (QA) Manager independently conducted Technical Systems Audits (TSA) of the laboratory tests at Moss Landing Marine Laboratories on June 25-28, 2017, the two field tests (the Monterey Bay surface mapping test during July 11-12, 2017 and the Chesapeake Biological Laboratory test during September 5-7, 2017), and data quality reviews of the reference data sets from all tests.

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 was 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 which 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 culture. 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 13. A total of 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 13. Summary of the number of reference samples collected at each site and the corresponding number of laboratory analyses.

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 - *T*he 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.

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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
Reliable Instruments for an Unreliable World

Turner Designs appreciates and thanks Alliance for Coastal Technologies (ACT) for their evaluation of our PhytoFind *in situ* Algal Classification Tool, a somewhat difficult endeavor filled with tedious nuisances due to dealing with live algae. We also thank ACT for this chance to provide a few notes/reminders to help explain the noticeable differences between ACT generated data and PhytoFind estimates from many of the tests performed, lab and/or field.

Major differences in lab tests stem from the fact that CDOM levels were too high for accurate estimation of algal groups causing an overestimation of the Mixed algal group.

CDOM is one of the primary interference materials when detecting *in situ* algal fluorescence. PhytoFind has an automatic CDOM correction function that does a good job at correcting for this interference from CDOM. It would seem that this function was not enabled for many of the lab trials. The enabling of this function is relatively easy, but may have been a difficult thing to accomplish for some of ACT's test setups; it requires users to filter the source water and read and record the responses from that source water. Those responses are then stored and used to correct the fluorescence detected, removing the bias from any CDOM present in the sample, and ACT noted this on page 46,

"The contribution of Mixed algae was inversely proportional to the amount of CDOM, which may reflect the use of a single initial sample to blank for CDOM effects."

However, not enabling or setting up the CDOM automatic correction for lab trials was fortuitous in that it showed just how much of an effect there is from high CDOM levels, reinforcing the thought that there is a need for setting a CDOM filtrate blank for all users working in CDOM-rich environments.

We also would like to note that when working with living organisms you could expect a great deal of variability, as results are never clear-cut due to the many factors affecting these organisms. Although we should be close to actual result, it is still only an **estimate** of what we think is happening based on the fluorescence detected.

Overall, we are satisfied with the performance of the PhytoFind and expect it to be a great tool for all users who are attempting to classify algal populations, whether it be for monitoring for a specific group of algae, simply keeping track of a changing population, determining the algal constituents in an algal bloom or correlating with bloom triggering events.

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