Protocols for Verifying the Performance of In Situ Chlorophyll Fluorometers

April 11, 2005

ACT PV05-01

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1. Background on ACT Technology Evaluations

Instrument performance verification is necessary to enable effective existing technologies to be recognized and so that promising new technologies can be made available to support coastal science, resource management and the long-term development of an Integrated Ocean Observing System. The Alliance for Coastal Technologies (ACT) has therefore been established to provide an unbiased, third party testbed for evaluating new and developing coastal sensors and sensor platforms for use in coastal environments.

The following protocols describe how ACT will verify the environmental performance characteristics of commercial-ready, in situ fluorometers through the evaluation of objective and quality assured data. The goal of this evaluation program is to provide technology users with an independent and credible assessment of instrument performance in a variety of environments. Therefore, the data and information on performance characteristics will cover legitimate information that users need. ACT will not simply verify vendor claims, but instead looks to the broader community to define the data and operational parameters that are valuable in guiding instrument purchase and deployment decisions.

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. ACT will avoid all potential pathways to picking "winners and losers". Therefore, although the following protocols will apply to all instruments evaluated, no direct comparisons will be made between instruments from different manufacturers and instrument-specific Verification Statements will be released to the public for each instrument type as a final report.

2. Introduction to Technology

As part of our service to the coastal community, ACT Partner Institutions and Stakeholder Council has chosen the performance verification of commercially available, in situ chlorophyll fluorometers as the second ACT Technology Evaluation. Fluorescence is the phenomenon of some compounds to absorb specific wavelengths of light and dissipate a portion of the absorbed energy by emitting, almost instantaneously, longer wavelengths of light. Chlorophyll *a* naturally absorbs blue light and upon blue excitation fluoresces red light. Fluorometers detect chlorophyll *a* by transmitting an excitation beam of light in the blue range and by detecting the light fluoresced by cells (or photosynthetic accessory pigments that transfer energy to chlorophyll *a*), in a sample in the red range. Generally, this fluorescence is directly proportional to the concentration of the chlorophyll. However, the amount of fluorescence emitted per unit chlorophyll can vary greatly in nature.

Chlorophyll measurements are widely used by resource managers and researchers to estimate phytoplankton abundance and distribution and can be used as a tool in assessing eutrophication status. Chlorophyll is also the most important light-capturing molecule for photosynthesis and is an important parameter in modeling primary production. These data are

used for numerous industrial applications as well, including water quality management, water treatment, ecosystem health studies, and aquaculture. There are various techniques available for chlorophyll determinations, including spectrophotometry, bench-top fluorometry and high performance liquid chromatography (HPLC) using samples collected on filters and extracted in solvent. However, chlorophyll measurement by in situ fluorescence is widely accepted for its simplicity, sensitivity, versatility, and economical advantages.

In situ fluorometers are designed to detect chlorophyll *a* in living algal and cyanobacterial cells in aquatic environments. The excitation light from the fluorometer passes through the water and excites chlorophyll within the living cells of the algae present. As light absorption by chlorophyll and its accessory pigments is the initial biophysical event driving photosynthesis, several factors make in situ fluorescence monitoring of chlorophyll a semi-quantitative measure at best. Environmental conditions, photoplankton community composition, physiological status, cell morphology, irradiance history and the presence of interfering compounds all play a role in altering the relationship between fluorescence and the concentrations of chlorophyll *a*. Interfering materials can compete with light absorption or change the optical path of fluoresced light and includes other plant pigments, degradation products and dissolved organic matter. Even with these diverse natural constraints, in situ fluorescence in a variety of deployment modes does supply valuable information on the relative temporal and/or spatial distribution of chlorophyll concentrations in the water column and under similar conditions correlates well with extracted chlorophyll *a* samples.

3. Objectives and Focus of Fluorometer Performance Verification

ACT has performed a customer needs and use assessment for in situ chlorophyll fluorometers. Scientists, resource managers, and other users of these technologies were asked to respond to a questionnaire regarding their current use or application of these instruments, limitations or problems with their current in situ fluorometer, and the important parameters they use when selecting a fluorometer. The results of this assessment were used to identify the main applications and key parameters that ACT will evaluate in this Technology Verification.

Almost equal numbers of respondents to our needs and use assessment indicated in situ fluorometers were commonly deployed on remote platforms in estuarine and near shore environments and used in profiling applications, typically down to at least 100 meters depth. Therefore, the present performance verification will focus on these two applications. It was also clear from the user survey that accuracy, precision, range (i.e., detection limits), and reliability are the most important parameters guiding instrument selection decisions. Given that in vivo or in situ fluorometry is a relative measurement with no absolute "true value" reference (see discussion above), accuracy in the measurement of chlorophyll in vivo cannot be determined directly. Regardless, much of the variation in fluorescence as a measure of chlorophyll is due to physiological and taxonomic factors that have nothing to do with any particular instrument. Therefore, a surrogate for accuracy will be used in this Performance Verification; response linearity or stability of the response/calibration factor to a defined reference (see below). Protocols were developed with the aid of manufacturers and Technical Advisory Committee to evaluate these specific areas. Complete needs and use assessment reports can be found at www.act-us.info/customer_needs.php.

3.1. Parameters to be Verified

Because of the inherent limitations of in situ flourometry and the inability to control various factors that can impact the data during field tests; response linearity, precision and range will be determined in the laboratory only. Field tests will focus on reliability/stability and the ability of the instrument to track natural changes in chlorophyll concentrations.

- **Response Linearity** – Stability of a predetermined response or calibration factor, computed as: (fluorometer measurement in sample solution – fluorometer measurement in blank solution) / [reference standard] over a range of reference standard concentrations. As relative fluorescence is temperature dependent, response factors will be quantified in the laboratory for each test temperature and the influence of reference dye and algal concentrations, varying standard turbidity concentrations, and light conditions will be assessed.
- **Precision –** Precision is a measure of the repeatability of a measurement. Instrument precision will be determined by calculating the coefficient of variation (STD/Mean x 100) of 30 replicate fluorometer measurements at 3 different reference dye concentrations and a fixed temperature in the laboratory.
- **Range** – Range or detection limit is a measure of the minimum and maximum concentration of specific reference dyes and in vivo chlorophyll *a* the instrument can accurately (see definition above) measure. Range and linearity will be determined on a dilution series of dye and algal concentrations in water under total darkness.
- **Reliability** – Reliability is the ability to maintain integrity or stability of the instrument and data collections over time. Reliability of instruments will be determined in two ways. In both laboratory and field tests, comparisons will be made of the percent of data recovered versus percent of data expected. In field tests, instrument stability will be determined by pre- and post-measures of blanks and reference dyes to quantify drift during deployment periods. Comments on the physical condition of the instruments (e.g., physical damage, flooding, corrosion, battery failure, etc.) will also be recorded.

4. Summary of Basic Verification Approach

The protocols are based on an amalgamation of protocols for sensor calibration and testing provided by the manufacturers participating in this ACT Performance Verification and the Technical Advisory Committee. Initial generic protocols were further refined through direct discussions during an ACT Fluorometer Performance Verification Workshop held on 9-11 March, 2005. Participants of this workshop included ACT Headquarters Staff, ACT Partner Institution Technical Coordinators, ACT Quality Assurance Manager, a Fluorometry Technical Advisory Committee, and representatives from each of the participating manufacturers. It was decided that the protocols will follow a format that:

- employs a reference dye and extractive chlorophyll *a* analysis through HPLC as the standard of reference for determining instrument performance characteristics,
- includes controlled laboratory tests, and
- includes field tests to evaluate performance under a variety of environmental conditions.

Qualified personnel affiliated with ACT will conduct all tests. All personnel involved in this verification exercise will be properly trained on use of instruments by manufacturer representatives and on a standardized water sampling, storage and shipping method. HPLC chlorophyll analysis will be based on established operational methods by a selected ACT Partner Institution with the proper skills and equipment (specifics provided below).

All numerical data will be recorded to three significant digits where appropriate. Instrument output will be blank adjusted and reported as:

- mV,
- digital counts,
- relative fluorescence units (RFU), and/or derived chlorophyll *a* values in µg/l (micrograms per liter).

Laboratory results will be presented as:

- means, standard deviations, and number of replicates (n) of instrument measurements in derived µg/l of Chlorophyll *a*;
- means, standard deviations, and number of replicates of corresponding reference dye concentration (µmolar or $O.D._{654nm}$) or µg/l of Chlorophyll *a* from extractive HPLC analysis; and
- the ratio of a fluorometers raw data output (mV or RFU) to corresponding reference dye concentration or µg/l of Chlorophyll *a* from extractive HPLC analysis of water samples.

Field data will be presented as:

- means, standard deviations, and number of replicates (n) of instrument measurements in derived µg/l of Chlorophyll *a*;
- means, standard deviations, and number of replicates of µg/l of Chlorophyll *a* from extractive HPLC analysis of water samples over time or depth;
- the ratio of raw data output (mV or RFU) to µg/l of Chlorophyll *a* from extractive HPLC analysis over time or depth; and
- associated physical conditions (e.g., temperature, salinity, TSS, ambient irradiance) over time or depth.

We acknowledge that the range of field environments is constrained due to testing and time limitations, but locations are widely geographically distributed and summer field conditions will have significant biofouling pressure.

Raw values from test instruments will be converted to produce derived chlorophyll *a* concentrations using the specific calibration factors that individual manufacturers would suggest to any of their customers. However, because of logistic constraints on this ACT Performance Verification, only data from two sets of environmental reference samples will be made available for instrument correction. Immediately before and after field performance testing periods, one water sample will be taken at each test site, divided into three replicates, and analyzed using the extractive HPLC method described below (which provides insight on abundances of basic taxonomic groups). These values and the corresponding first and last values taken by the instruments in the field will be made available for correcting data using equations or methods provided by manufacturers. The values used for corrections will, however, be excluded from the data presented as part of the Performance Verification.

The goal of this Performance Verification is to test the same model instruments in the laboratory, in a moored application, and in a profiling application. It is also preferred to evaluate instruments incorporated in stand-alone packages, which include features such as data logging, data transformation/conversion equations, independent power, and biofouling prevention. However, in some cases, certain test instruments will only be tested in one type of field application (if they are designed and sold exclusive for one particular use) and some independent sensors will be incorporated into other associated equipment (e.g., datalogger, CTD) owned and operated by ACT Partner Institutions.

A total of four sensors of each particular model will be evaluated during this verification. For the laboratory exercise, one fluorometer of each model will be randomly selected for testing. The moored field tests will use all four instruments, conducted over the course of two separate evaluation periods. The first will include the test instruments deployed simultaneously at four ACT Partner sites followed by a second set of evaluations at the remaining ACT Partner sites (seven total moored deployments). Profiling field tests will take place before or after the moored tests at two ACT Partner sites.

4.1. HPLC Analysis of Extractive Chlorophyll

The HPLC method to be used for chlorophyll *a* analysis follows that of Zapata et al. (2000, MEPS 195:29-45). This method utilizes a C8 reverse-phase column which delivers separation of monovinyl and divinyl chl *a*, and includes the addition of a pyridine-based, mobile phase modifier that provides separation of the most common polar chlorophylls; these include chl c_1, chl c_2, chl c_3, Mg 3,8 pheoporphyrin a_5, and chlorophyllide *a* (chlide *a*). The Welschmeyer lab at Moss Landing Marine Laboratories (MLML, the West Coast ACT Partner Institution) will be conducting the chlorophyll *a* analysis for this ACT Performance Verification. This laboratory group has used this method over the last three years with great success, concluding that the slightly longer run time (ca. 40 min per sequential injection) is outweighed by the increased peak resolution relative to previous C8 methodologies (Culley and Welschmeyer 2002, L&O 47: 1508-1513).

This HPLC system includes a Varian ternary gradient pump (operated in binary mode as per Zapata et al. 2000), with in-line detectors including a Thermo Separation Products Spectra Focus VIS absorbance detector (operated at 440 nm; spectral scans available when needed), Kratos 950 filter fluorometer fitted with broadband red and blue filters to provide sensitivity to all chlorophyll-derived fluorescent products. Solvent A (methanol:acetonitrile:0.25M aqueous pyridine, 50:25:25 v:v:v) and solvent B (methanol:acetonitrile:acetone, 20:60:20) are run at 1 mL min^{-1} according to gradient (a) of Zapata et al. (2000). Samples are injected by a Gilson 201 autosampler, with continuous sample cooling (10ºC) and sample dilutions (2:1 v:v, sample:H_2O) made just prior to injection (to improve shape of early-eluting peaks). Data will be collected on a computer controlled peak integrator for pigment quantification. Sample concentrations will be determined from purified chl *a* standards injected in series with each autosampler run.

We anticipate that most coastal samples will dominated by monovinyl chl *a*, with little, or no divinyl chl *a*; the HPLC method above will adequately monitor the potential presence of divinyl chl *a* should prochlorophytes be present. In coastal systems, particularly during diatom blooms, quick filtering and freezing of all samples to avoid enzymatic degradation of chl *a* to chlide *a* is critical. Participating sampling teams will be trained to be aware that potentially high chlorophyllase activity in diatoms (also in green algae) can convert more than half the original chl *a* to chlide *a* if wet filters are left at room temperature for several hours. Total chl *a*

concentrations as the molar sum of chl *a* and chlide *a* will be reported (in addition to mg/L), thus accounting for possible artifactual production of chlide *a*. However, chlide *a* production will be avoided whenever possible.

All samples from Partner sites (other than MLML) will be frozen in liquid N_2 and shipped by overnight courier in liquid N_2 dry shippers to MLML for analysis in two shipments upon completion of deployments. Chain of custody (COC) forms will accompany all samples during shipping (See section 7.3.3). Samples will then be extracted in N_2-purged 90% acetone overnight, followed by autosampler HPLC processing commencing the following day. All extracts will be simultaneously analyzed by standard fluorometric technique (Welschmeyer 1994, L&O 39: 1985-1992) to complement HPLC assays described above.

4.2. Laboratory Tests

Laboratory tests of response linearity, precision, range, and reliability will also be conducted at MLML. As the goal of the laboratory tests is to assess performance of the fluorescence detection systems rather than biologically based variation in chlorophyll fluorescence, an inert fluorochrome will be employed as the reference standard. Constraints on dye selection are that it be (a) water soluble, (b) have absorbance and fluorescence emission properties that overlap the optical specifications of the in situ fluorometer systems and (c) that its absorbance and fluorescence emission properties be stable (at minimum quantifiable) under a range of water conditions. Basic Blue 3 (BB3, C.I. 51004, CAS 33203-82-6, M.W. 359.9) meets these specifications as fluorometric reference standard and critically, has been employed as a wide wavelength range (220 -700nm) quantum counter for correction of fluorescence emission spectra (Kopf and Heinze 1984 *Anal. Chem.* 56, 1931=1935). BB3 is readily soluble in both deionized and sea-water (>>1 mg / mL or > 2.8 mM) without substantial shifts in absorbance properties (λ_{max} = 654, $\varepsilon_{M,654}$ = 88954, λ_{em} = 661 nm) and is inexpensive ($0.45 /gm) making it feasible to use in immersion bath tests. Based on these dye features, laboratory tests evaluating in situ fluorometer performance in different water conditions will in part involve the comparison of fluorometer output in the presence of varying concentrations of BB3 under different defined reference conditions (e.g. 15^{o}C, 1 µg / mL BB3, dark). The optical behavior of BB3 in the selected water conditions (e.g. temperature dependence) will be monitored independently by absorbance and fluorescence spectroscopy using calibrated research grade instrumentation (TU 1901 UV/VIS dual beam spectrophotometer and a Spex Fluorolog scanning spectrofluorometer). BB3 samples will be collected via pipette from the center of the test baths and placed directly in to a cuvette for analysis.

At the request of the participating manufactures and on recommendation of the scientific advisory panel, the dye Rhodamine WT (RWT, λ_{max} = 497, λ_{em} = 523 nm) will be used in a limited number of independent test conditions to permit cross calibration of BB3 and RWT fluorescence signals.

The various conditions below will be produced in well-mixed (submersible circulating pumps), temperature controlled (monitored at two locations in each bath) water baths where instruments will be submerged for testing. Instrument output will first be "calibrated" to BB3 and/or RWT concentration under standard reference conditions by immersion in one or two-point standardization solutions as suggested by each manufacturer.

Although field tests will include instrument deployments under varying salinity conditions, it was decided that salinity would not be tested as a variable in the laboratory

evaluations described below. All laboratory tests, except for those with live algae, will be conducted in filtered, deionized water.

Response Linearity – For the linearity or stability tests, a mean and standard deviation of 5 instrument readings at 1-minute intervals for each test condition will be collected after the instruments are allowed at least 30 minutes to equilibrate. This instrument mean and STD will be compared to the mean and STD of 5 water samples BB3 samples collected at the same 1-minute intervals and quantified by absorbance spectroscopy. Test baths will filled with DI water (to provide a baseline or zero response factor), held in the dark. At least five concentrations of BB3 will be tested over the range of 0-15 µg / mL (e.g. 0, 0.05, 0.5, 1, 5 and 15 µg / mL) and temperature (4, 15 and 32 ^{o}C) will be varied to produce a matrix with 18 independent conditions for evaluating response linearity.

Precision – Precision tests will be conducted simultaneously by monitoring the variance of instrument fluorescence signal over 30 consecutive measurements at 1 minute intervals in a selected subset of controlled bath conditions in the test matrix described above. Minimally instrument precision will be determined at each dye BB3 concentration tested in the reference water temperature of 15 ^{o}C. Precision of the BB3 dye concentration assay will be measured similarly. These tests will be repeated using RWT as the reference dye.

Detection Range – The experimental matrix above will enable determination of the linear detection ranges at each test temperature. Limit of detection will be computed as: (Mean + 3 S.D. of blank readings) and upper detection range will be determine as either the dye concentration causing saturation of instrument output or a greater than 50% decline in response factor. Only tests conducted in dark conditions will be used to determine detection limits of the instruments. These range estimates will then be independently tested at a reference temperature of 15^{o}C by monitoring instrument output over a low range of BB3 concentrations (e.g., 0.001, 0.005, 0.01 and 0.025 µg / mL) and very high levels of BB3 (e.g., 5, 10, 15 and 20 µg / mL). The exact BB3 concentrations tested may vary depending on instrument response or gain settings. These tests will be subsequently repeated at 15^{o}C using RWT as the reference dye.

Light and Turbidity – Sensitivity to ambient light and water clarity will then be assessed by exposing the test instruments to high light (>200 umol/m/s PAR) using 500W halogen worklights and varying turbidity levels. Instruments will be placed in a bath at 15^{o}C with 1 µg / mL of BB3 and response factors, measured as described above, obtained in darkened conditions and under high irradiance (ca 200 µmol quanta /m^{2} / s, near sensor heads) conditions will be compared. Two different levels of turbidity (very low, set by the water source, low and high, 400 NTU max.) will then be produced by adding combinations of Formazin and colored dissolved organic matter (CDOM, filtered coffee will be used for simplicity). The response linearity test repeated under high light and in the dark. Turbidity test conditions will be quantified by a benchtop turbidity sensor in NTU. CDOM will be determined by absorbance spectroscopy on filtered samples (see below).

In vivo Chlorophyll Detection – Instruments characterized for their BB3 fluorescence response will be evaluated for their response linearity and detection limit/range for in vivo chlorophyll fluorescence under standard reference conditions (32 ppt, 15^{o}C, dark). This will

permit comparison of chlorophyll fluorescence in terms of the BB3 based RFU for assessment of instrument drift. The diatom *Thalassiosira pseudonana* Clone 3H (CCMP 1335) will be grown in a batch culture (75 µmol quanta m^{-2} s^{-1} PAR 40W fluorescent bulbs, continuous light at15^{o}C) with f/2 enriched seawater to mid-log phase (determined by cell counts), then propagated in semi-continuous culture at ca. 0.25 /d to maintain cell concentration at a level required for the dilution test. Instruments will be equilibrated as described above and allowed to collect 10 "blank" samples. Dark adapted (>1 h) 3H cells will be added and the measurements repeated. Chlorophyll samples will be taken as described above bracketing the measurement period (1st sample, 5th sample, l0th sample). 3H cells concentration will be sequentially increased to provide a range of target chlorophyll concentrations (e.g. 0, 100, 1000, 10000, 100000 cells / mL rapid proxy for chlorophyll added to the system).

Finally, instrument reliability in the laboratory will be determined by comparing percent of data recovered versus percent of data expected. Comments on problems or instrument failures will also be recorded.

4.3. Field Tests

Approximately 7 days prior to their particular field deployment, each test site will collect 10 independent water samples over a one-hour period at the location where field tests will occur. These samples will be processed, handled, and shipped to MLML for HPLC analysis as described elsewhere in this document. This preliminary exercise will help characterize the various test environments, refine the sampling process, and assure that the HPLC analytical methods proposed are appropriate for all the conditions that will be tested. Each site will also perform an assessment on how much water must be filtered to capture an appropriate quality chlorophyll *a* for extraction.

Instrument reliability in both a moored and profiling application will be determined by comparing percent of data recovered versus percent of data expected. Comments on problems or instrument failures will also be recorded.

4.3.1. Moored Deployment

In situ evaluations of instrument performance in a moored application will be conducted at each of the seven ACT Partner Institution sites (site descriptions below). One fluorometer from each manufacturer will be deployed for four continuous weeks (26 to 28 days) at each site. They will only be removed from the water after the test period is complete or in the event of a problem such as a weather event that could jeopardize the safety of the instruments. Because each manufacturer will provide only four total instruments, two sets of consecutive field tests will be run. Instrument packages will, however, be returned to manufacturers for a maximum of 3 weeks for reconditioning and calibration in between the two sets of field tests and prior to field profiling tests (see below)

Instrument Setup - Prior to deployment, all instruments will be setup and calibrated at the field sites as suggested in individual manufacturer manuals. Fluorometers will then be programmed to record data every 15 minutes during the entire field deployment and their internal clocks set to local time using www.time.gov as the time standard. A photograph of each individual fluorometer and the entire instrument rack will be taken just prior to deployment and just after recovery to provide a qualitative estimate of biofouling during the field tests. Finally,

all instruments will be placed in a bath of deionized water (as a blank) and then a container of 1 µg / mL of BB3 and one with RWT as the reference standards (from stocks prepared and distributed by MLML as described above) both before and after deployment and allowed to make three readings of fluorescence, as an estimate drift over time. If possible, the post blank and reference standard measurements will be made both with the instruments taken directly from the field and again after biofouling has been removed from the optical surfaces and instrument cleaned.

Deployment Rack - All instrument packages will be deployed on a single, box-shaped rack that allows all sensor heads to be at the same depth, and with each manufacturer's instrument side by side. Instrument sensor heads will be deployed at the closest proximity that their designs will allow but far enough apart to prevent cross interference (as separation distance of at least 1 instrument diameter). The rack will be deployed so that all of the fluorometers remain at a fixed depth of 1 m below the water surface (using a float system or on a floating dock). A calibrated CTD package will also be attached to the rack at each test locale and programmed to provide an independent record of conductivity and temperature at the same depth and the same 15-minute intervals as the test instruments. The sensor rack design will also be standardized as much as possible from site to site. However, physical conditions at particular sites may require specialized modifications. Each site will maintain a PAR cosine sensor to continuously monitor surface irradiance history at the deployment site. Water clarity will be determined at least once a day during sampling using a black and white 200 mm secchi disc.

Sampling Schedule - At the initiation of deployment, and immediately prior to termination of the field deployment, three consecutive water samples will be drawn >1 hour before dawn, corresponding to instrument sampling points, and processed as above. The chlorophyll *a* values determined for these samples will be used as reference field calibrations for each instrument. Subsequently, during the first day, last day, and at some point near the middle of the four-week deployment (between the 8^{th} and 18^{th} day of deployment), each site will collect water samples for extractive HPLC every 3 hours for a 24-hour period (coinciding with test instrument readings) for a total of three full diel cycles. During the remainder of the moored deployments, each test site will take 2 Van Dorn water samples every weekday (M-F), each timed to again correspond to the instrument sampling time. The timing of water sampling on days other than first and last of the field tests will be left up to the individual site with the goal of capturing natural daily variations in chlorophyll levels. However, one sample must be taken during day light hours and the second either >1 hour prior to sunrise or >2 hours after sunset. Furthermore, any specific reference water samples will only be taken when all test instruments are exposed to the same basic light environment (i.e., all uniformly shaded or uniformly lit).

Water Samples - A standard 2-l Van Dorn bottle will be used at each field test site to collect water samples for extractive HPLC chlorophyll *a* analysis. These samples will be used as the standard for examining instrument performance/stability through time. The bottles will be lowered into the center of the sensor rack, at the same depth and as close as physically and safely possible to the fluorometers. The bottle will be triggered to close at the same time as instrument sampling, to ensure that the same water mass is being compared to in situ chlorophyll *a* concentration. The entire water sample will be emptied through the bottom of the Van Dorn bottle into a light protected 4 or 5-l carboy (using a large funnel) and transported to the laboratory at ambient in situ water temperature for further subsampling and processing. Prior to subsampling the whole water sample is homogenized by carefully inverting the carboy 3 times. Each subsample is quickly drawn from the carboy by pouring and collected in a graduated

cylinder. Under low light conditions, four subsamples (volume to be determined for each site) will be filtered and stored for chlorophyll *a* analysis as described above. Three of the samples will be shipped to MLML for analysis and one will remain at the test site as a backup sample (see Quality Assurance Plan for chain of custody details). A fifth and sixth subsample (volumes to be determined for each site) will also be taken from the original Van Dorn collection and carboy, where the filtrate of one subsample will be used to determine CDOM (see below) and the other analyzed for total suspended solids (TSS, see below). CDOM and TSS will be used as estimates of turbidity at the individual test sites during testing. The Van Dorn bottle will be wiped with a clean cloth and rinsed with copious DI water (>5 rinses) between uses.

Filtering - All chlorophyll *a* samples from each ACT Partner test site will be filtered on 2.5 cm Whatman GF/F membranes immediately after collection (<30 min). Sufficient volume of sample water will be filter to get visible color on filter surface and the precise volume filtered (400 mL maximum) for each sample recorded. All filtrations will be done in low light (i.e. not in front of window, away from sunlight), with low vacuum pressure (<5 in Hg). As soon as fluid runs through the filter, it will be removed, folded in half with the sample on the internal surface (with the vacuum still on), placed in cryovials (one filter/vial) and immediately frozen by immersing into liquid N_2. Quick-freezing in liquid N_2 improves extraction efficiency and provides excellent long-term storage with no degradation (Bidigare et al. 2005, Analysis of pigments by high-performance chromatography. In R. Andersen (ed) Algal Culturing Technique, Academic Press).

Cleaning - Filtration apparatus and sample storage vessels will be cleaned between use by wiping with a clean cloth and copious rinses (>5) with DI water. Once per week (typically Fridays after sampling) filtration apparatus will be wiped, rinsed and soaked overnight in a 2% solution of Micro-90 or equivalent detergent, followed by a second round of copious rinsing with DI water.

4.3.2. Profiling Deployment

In situ evaluations of instrument performance in a profiling application will be conducted at only two of the ACT Partner Institution. CILER/University of Michigan will conduct one set of profiling tests in freshwater of the Great Lakes. A second set of tests will be run in marine coastal waters by GoMOOS/University of Maine. At each of the Partner profiling test sites, three to five independent profiles will be conducted at varying locations during a single cruise, where simultaneous electronic measurements and discrete samples are collected from the ship at six discrete depths throughout the water column. The basic design will to collect two reference samples in the surface mixed layer, one at the chlorophyll/particle maximum (if present) and three below the pycnocline including the clear water minimum, in order to capture the maximum variation in chlorophyll levels. Each discrete depth will be sampled in replicate with two independent bottle collections. The exact depth locations will be determined on the basis of water column depth and the observed flurometry profile obtained in real-time during the downcast of the rosette system. An independent, real-time recording fluorometer will be incorporated into the CTD package on the sampling rosette so basic depth profile of chlorophyll can be identified as the test instruments are being lowered to the maximum sampling depth. The data from the independent real-time fluorometer will only be used as an aid in selecting appropriate sampling depth designs and will not be distributed or used in any comparisons. The rosette will be lowered and raised at the standard rate of between 0.25 – 0.5 m/sec and the data collected by test instruments will be presented for both down- and up-casts.

Instrument Setup - Each of the profiling test sites will randomly select one of the instruments provided by each manufacturer for the ship-based testing. Each of the profiling test sites will test a set of instruments, as determined by the participating manufacturers, in profiling mode. Prior to deployment, all instruments will be calibrated as suggested in individual manufacturer manuals and with a blank, BB3 and RWT at the field sites (as described above). The fluorometers will then be programmed to record data at between 1-5 Hz for the profiling tests, depending upon the manufacturer's recommendation, and their internal clocks set to local time using www.time.gov as the time standard. The instrument from each manufacturer will then be mounted within a modified 12-bottle profiling rosette so that all fluorometers and bottles measure and sample near the same depth as physically possible. A standard and calibrated CTD package and PAR sensor will also be attached to the rosette and programmed to provide an independent record of conductivity, temperature and light during each instrument sampling event.

Water Samples - Water samples for extractive HPLC analysis of chlorophyll *a* will be collected only on the up-cast. At each of the selected depths, the rosette will be paused for 1 minute to ensure that all fluorometers have equilibrated to those conditions and that a significant number of readings at the specific depth have been recorded. After the one minute delay, two Niskin bottles will be fired at each depth and sampling time, bottle numbers, depth, and profile number will be recorded on the field data log. For any of the test fluorometers that cannot be connected directly into the CTD logging unit of the rosette, profiling data will be internally logged and then matched up to the other fluorometer by matching the time-stamp. The minute sampling intervals at each depth will help ensure that appropriate data are extracted for comparison.

Water samples will be processed immediately upon retrieving the rosette on deck. For each sample, the entire contents of the Niskin bottle will be drained into an opaque sampling carboy, as with the moored deployment procedure, to minimize any heterogeneity in particle distributions due to settling. Samples will be collected under shade to minimize light exposure and immediately taken into a shipboard laboratory and processed using the same protocols as defined for the field mooring deployments.

4.4. Water Quality Characterization

4.4.1. Colored Dissolved Organic Matter (CDOM)

Sample Preparation – Approximately 40 ml of sample water will be filtered and used to rinse the sides of the flask and the 50 ml BD Falcon storage centrifuge tubes), and then discarded. Approximately 45 ml of the CDOM designated subsample will be subsequently filtered using 47 mm GF/F filters (0.7 μm pore size) with low vacuum pressure (<5 in Hg). The filtrate will be place in the centrifuge tube, capped, wrapped with Parafilm, labeled, and stored in a refrigerator (4° C) until analysis. All samples will be shipped to MLML on ice for analysis using a calibrated laboratory-grade spectrophotometer.

Sample Spectrophotometric Analysis – The sample and 200-300 ml of MilliQ will be equilibrated to room temperature (failure to match the blank and sample temperature results in an artifactual feature in the spectrum at long wavelengths). The spectrophotometer will be allowed to warm up (30-40 min) before scanning and the scanning set for 1-2 nm intervals, with a 4-6 nm slit width.

The blank will be set with MilliQ water in cuvettes in both the sample and reference positions (dual-beam instrument) or by setting 0 and 100% transmission with a MilliQ blank (single-beam instrument). Matched 10 cm quartz or optical glass cells will be used for a dual-beam spectrophotometer and a single cell in a single-beam spectrophotometer. A rescan of the blank will be run to verify that the instrument has not drifted and to define the amplitude of noise.

The sample will be mixed by rocking the centrifuge tube before decanting the sample. The sample cuvette will be emptied and rinsed with 10-15 ml of sample prior to filling with sample and checked to ensure that there are no air bubbles in the light path when filled. Scans will be run between 250 and 800 nm (quartz) or 350 and 800 nm (optical glass) and electronic files will be saved for each sample. The cuvette will then be rinsed with MilliQ between samples. A MilliQ blank will be run between every 5th sample. When using a single-beam spectrophotometer, it will be reset at 0 and 100% T. For dilute samples (those that are not significantly yellow in color when viewed down the 10 cm pathlength of the cuvette or have OD < 0.2 at 400 nm), triplicate scans of each sample will be run and average the scans to reduce the effect of noise.

Parameterizing Absorption – The absorption spectrum of CDOM follows an approximately exponential decline in the visible (400-700) wavelengths. Absorption from optical density will be calculated by subtracting the optical density at 750 nm to correct for residual scattering and converted as:

$$a[\lambda] = \frac{2.3\left(OD[\lambda] - OD[750]\right)}{d} \qquad (1)$$

where $a[\lambda]$ is absorption (m^{-1}) at wavelength λ (nm), OD is optical density (dimensionless), and d is pathlength (0.1 m).

Because the magnitude of the parameters that describe the CDOM fit are dependent on the range and means of the curve fitting, it is critical to employ the same protocol. A non-linear fit over the range 400 – 700 nm (Equation 2) will be used. This equation minimizes the effect of the unfavorable signal:noise ratio at long wavelengths on the goodness-of-fit at the wavelengths of most interests (i.e. the excitation wavelengths in the 400 – 500 nm range); is less sensitive to thermal artifacts at long wavelength; and is less sensitive to the range of wavelengths used than the linear fit (Equation 3).

$$a[\lambda] = a[400]e^{(-S\lambda)} \qquad (2)$$

$a[\lambda]$ is absorption (m^{-1}) at wavelength λ, $a[400]$ is absorption (m^{-1}) at the anchor wavelength of 400 nm, and S is the spectral slope (nm^{-1}). Note that wavelength must be expressed as $\lambda - 400$ before fitting for the anchor value to be at 400 nm.

An alternative for those without a non-linear fitting package is to log-transform the absorption data and fit to the linear equivalent.

$$\ln\left(a[\lambda]\right) = \ln\left(a[400]\right) - S\lambda \qquad (3)$$

Note that the anchor wavelength must be expressed as the anti-log for reporting. Because the linearized fit is sensitive to the dispersion due to noise at long wavelengths, this is best fit only for the interval 400-500 nm.

The parameter estimates and standard errors for $a[\lambda]\ S$ and the value of R^2 will be reported.

4.4.2. Total Suspended Solids (TSS)

TSS is the retained material on a standard glass filter pad after the filtration and drying of a well-mixed sample of water, with the results expressed in mg/L. The methods to be used are based on APHA Method 208D (1975), USEPA Method 160.2 (1979), and Etcheber, H. 1981, *Journal de recherche oceanographique*, 6:37-42.

Personnel from each test site will be trained on this standardized method and will conduct a minimum of five test TSS analyses with water collected at their moored deployment site prior to field tests to help determine the appropriate sample size to yield <200 mg of residue.

Pre-collection Preparation -

1. Sequentially number Whatman 47 mm GF/F filters (0.7 µm pore size) along the outside edge, where the sample will not pass through, using a fine-tipped permanent marker.
2. Pre-rinse both sides of filters prior to use.
3. Dry filters at 60 - 65°C overnight (18- 24 hours).
4. Weigh filters (in grams) to 4 decimal places to obtain pre-weight. Pre-weighed filters are stored in sealed containers until ready for use.

Sample Collection and Handling –

1. Pre-rinse the filter with 0.5 M ammonium formate to wet the entire filter pad and then put filter cone in place. Pre-rinsing will help prevent the formation of a salt "halo" that tends to creep along toward the outer edge of the filter, as a dry filter is wetted at its center.
2. Filter a known volume of water through the filter pad and rinse filter with 0.5 M ammonium formate to remove salts (both the ammonium formate rinses can be excluded for freshwater samples).
3. Fold pad in half, sample inside, and place in a sealed container or aluminum foil pouch.
4. Freeze filter pads for storage.

Analytical Procedure –

1. Dry filters at 60 - 65°C overnight (18-24 hours). Allow samples to cool to room temperature in dessicator.
2. Weigh filters and record weights (in grams).
3. Subsample a portion of the filters and replace them in the drying oven for a minimum of 1 hour.
4. Re-weigh subsampled filters. If there is >0.5 mg weight loss between the first and second weight of the subsampled filter pads, then all filter pads should be re-dried and re-weighed.
5. Repeat steps 3 and 4 as necessary.

Calculation of TSS –

TSS concentration is calculated using the following equation:

$$mgTSS/L = \frac{(W_{post} - W_{pre})\,x1000}{V} \quad (4)$$

where:
W_{post} = dry weight of filter pad after filtering (g),
W_{pre} = dry weight of filter pad before filtering (g), and
V = volume of water filtered (L).

4.4.3. Ancillary Data

In conjunction with each water sample collection, each deployment site will also record site-specific conditions. The following information, logged on standardized datasheets, will be transmitted on a weekly basis to the ACT Chief Scientist, for data archiving and ACT personnel performance QA/QC:

- Date and time (local) of water sample collection.
- Light attenuation by 200 mm diameter Secchi disk depth reading, <3 meters from the deployment rack and when possible in direct sun light.
- Ambient light in air by PAR sensor <50 meters of the deployment rack (recorded continuously during deployment).
- Weather conditions (e.g., haze, % cloud cover, rain, wind speed/direction) and air temperature at time of water sample collection.
- Recent large weather event or other potential natural or anthropogenic disturbances.
- Tidal state and distance from bottom of sensor rack at time of water sample collection.
- Any obvious problems or failures with instruments.

5.0. Verification Schedule (planned dates but may vary).

- The Final Verification Protocols and ACT Verification Contract will be sent to Manufacturers on April 11, 2005
- Signed contracts are due back to ACT Headquarters by May 6, 2005
- All instruments to be test will be delivered to MLML by May 14, 2005
- ACT Chief Scientist, Technical Coordinators, Quality Manager, and Manufacturer Representatives will meet at MLML for instrument use/operation/deployment, sample collection, storage and shipping training on May 18 – 21, 2005
- Selected ACT staff will conduct the laboratory verification tests on May 23 – 31, 2005
- All instruments will be delivered to the first four ACT test sites by June 3, 2005
- The first four 4-week moored deployment (SkIO, USF, MLML and UMich) and one profiling (UMich) verification tests will begin on June 6, 2005
- All instruments will be sent back to individual Manufacturers for reconditioning and calibration on July 4, 2005
- Instruments will be sent back from Manufacturers to the second set of three ACT test sites and received by July 25, 2005
- The second set of three 4-week moored deployment verification tests (CBL, UMaine and UHawaii) will begin on August 1, 2005
- All instruments will be sent back to individual Manufacturers on August 31, 2005
- One final set of set of reconditioned instruments will be sent for the final profiling verification tests (UMaine) on September 19, 2005

- Final set of profiling instruments will be sent back to Manufacturers on September 30, 2005.
- ACT Chief Scientist, Technical Coordinators, Technical Advisory Committee, and Quality Manager, will meet for 3 days to analyze results and evaluate the Verification processes in early November 2005
- ACT Verification Statements for each individual instrument will be drafted and sent out for review by, Technical Advisory Committee, Technical Coordinators, Quality Manager, Partners, and Stakeholders in early December 2005
- Final Verification Statements will be sent to Manufacturers on January 6, 2006
- One page comment letters from Manufacturers are due by January 13, 2006
- Final Verification Statements will be released to the public in February 1, 2006

6. Data Recording, Processing and Storage

This section describes methods employed during data recording, processing, and storage to minimize errors and assure high quality analyses in the Verification Statements.

6.1. Documentation and Records

A variety of data will be acquired and recorded electronically and manually by ACT staff in this verification test. Operational information and results from the reference method will generally be documented in a field/laboratory record book and on the data sheet/chain-of-custody forms (see below). An electronic copy of these raw data will be transferred to the ACT Chief Scientist weekly, who will store it permanently along with the rest of the study data.

The results from the test fluorometers will also be recorded electronically. Test instrument data will be logged by individual sensor packages (?) and will only be downloaded and analyzed upon completion of the four-week field deployments. Once collected, one copy of these data will reside at the corresponding ACT test facility and a second copy at ACT Headquarters and until the entire verification is finished. The table below summarizes the types of data to be recorded and the process for recording data.

Data to be Recorded	**Responsible Party**	**Where Recorded**	**How Often Recorded**	**Purpose of Data**
Dates, times of sampling events	Each ACT Partner	Field/laboratory record books/data sheets	Each reference sample collection and laboratory analysis	Used to organize/check test results; manually incorporate data into spreadsheets - stored in study binder
Test parameters (site conditions)	Each ACT Partner	Field/laboratory record books/data sheets	Each reference sample collection	Used to define site characteristics; manually incorporate data into spreadsheets - stored in study binder
Test parameters (ancillary data) CDOM and TSS	MLML ACT Partner	Laboratory record book/data sheets	At the conclusion of each analytical sample batch.	Used to define site characteristics; manually or electronically incorporate data into spreadsheets - stored in study binder

Test instrument calibration data	Each ACT Partner	Laboratory record book/data sheets	Start/end of test	Document correct performance of test instrument
Test instrument data - digital display - electronic output	Each ACT Partner	- Data sheets - Instrument data acquisition system (data logger)	After completion of the 26-day field deployments	Used as part of test results; incorporate data into electronic spreadsheets - stored in study binder
Reference analytical results	MLML ACT Partner	Laboratory record book/data sheets	At the conclusion of each analytical sample batch.	Used to check test results; manually incorporate data into spreadsheets - stored in study binder
Reference calibration data	MLML ACT Partner	Laboratory record books/data sheets	Whenever zero and calibration checks are done	Document correct performance of reference method
Performance evaluation audit results	ACT HQ	Laboratory record books/data sheets	At times of performance evaluation audits	Test reference method with independent standards/ measurements

6.2. Data Review

All data are to be recorded directly in the field/laboratory record book as soon as they are available. Records are to be written in water-proof ink, written legibly, and have any corrections initialed by the person performing the correction. Any corrections will be crossed out with a line (not blackened or white-out), and the correction made, with initials and date of correction. These data will include electronic data, entries in field/laboratory record books, operating data from the ACT Partner test facility, and equipment calibration records. Records will be spot-checked within two weeks of the measurement to ensure that the data are recorded correctly. The checker shall not be the individual who originally entered the data. Data entries shall be checked in general for obvious errors and a minimum of 10 percent of all records shall be checked in detail. Errors detected in this manner shall be corrected immediately. The person performing the review will add his/her initials and the date to a hard copy of the record being reviewed. The ACT Technical Coordinator (TC) will place this hard copy in the files for this verification test. In addition, data generated by each ACT Partner test site will be provided to the ACT Chief Scientist and reviewed before they are used to calculate, evaluate, or report verification results.

7. Quality Assurance/Quality Control

Technology performance verifications are implemented according to the test/QA plans and technical documents (e, g. Standard Operating Procedures) prepared during planning of the verification test. Prescribed procedures and a sequence for the work are defined during the planning stages, and work performed shall follow those procedures and sequence. Technical procedures shall include methods to assure proper handling and care of test instruments. All implementation activities are documented and are traceable to the test/QA plan and SOPs and to test personnel.

7.1. Analytical Laboratory Quality Control for HPLC

HPLC Reference Measurements shall have the following Quality Controls:

a. Perform a spike recovery (TBD)
b. Analyses of Blanks.
    a. Weekly analysis of blanks. These blanks will be collected weekly during sampling and should include:
        i. Field Blanks (from Carboy) – (see Section 7.2).
        ii. Sampler Device Blank – (see Section 7.2).
        iii. Filtration Blanks – (see Section 7.2).
    b. These blanks will be prepared at MLML and analyzed during the HPLC run weekly.
        i. Reagent Blanks
        ii. Extraction Procedure Blanks
        iii. Instrument Blanks
c. Calibration with Standards.
    a. Measure at least three different dilutions of the standard when analysis is initiated. Verify the standard curve daily by analyzing one or more standards within the linear range, as specified in the individual method. Results are reported which are in the range of standard dilutions used.
    b. Performance Evaluation Audit. Perform a verification of your calibration standard (see Section 7.3).
d. Control Charts. Three types of control charts are used in laboratories: a mean chart for **standards** - laboratory control standards or calibration check standard; a mean chart for background or reagent **blank**; and a range chart for **replicate** analyses.
e. Records of Standards and Chemicals: Records must be kept of all standards, solvents, and other chemicals used during analysis and extraction. This information must include: Name of Chemical/CAS#, Manufacturer, Lot#, the date received by laboratory, expiration date, the date the chemical was opened for use, and NIST-traceability. Material Safety Data Sheets must be on file in the laboratory and all safety and storage precautions related to those chemicals must be followed.
f. Blind Sample Run: Ideally, all samples should be run as "blind" samples – by the sample number only.

7.2. Analytical Laboratory Quality Control for BB3

BB3 Reference Measurements shall have the following Quality Controls:

a. Analyses of Blanks. Weekly analysis of blanks.
        i. Sampler (Cuvette) Blank.
        ii. Reagent Blanks (if necessary)
        iii. Instrument Blanks
b. Calibration with Standards.
        i. Measure at least three different dilutions of the standard when analysis is initiated. Verify the standard curve daily by analyzing one or more standards within the linear range, as specified in the individual method. Results are reported which are in the range of standard dilutions used.
        ii. Performance Evaluation Audit. Perform a verification of your calibration standard (see Section 7.3).

c. Analysis of Duplicates. Analysis of duplicate sample is effective for assessing precision, which is accomplished by analyzing 5% or more of the samples in duplicate (see Section 7.2).
d. Control Charts. Three types of control charts are used in laboratories: a mean chart for **standards** - laboratory control standards or calibration check standard; a mean chart for background or reagent **blank**; and a range chart for **replicate** analyses.
e. Records of Standards and Chemicals: Records must be kept of all standards, solvents, and other chemicals used during analysis and extraction. This information must include: Name of Chemical/CAS#, Manufacturer, Lot#, the date received by laboratory, expiration date, the date the chemical was opened for use, and NIST-traceability. Material Safety Data Sheets must be on file in the laboratory and all safety and storage precautions related to those chemicals must be followed.
f. Blind Sample Run: Ideally, all samples should be run as "blind" samples – by the sample number only.

7.3 Laboratory Test Quality Control

Both the test and laboratory reference instrumentation to be used in this verification test will be calibrated by the ACT TC at MLML according to the SOPs for the instrumentation prior to field deployment. Each TC for each instrument will maintain a calibration log. The logs shall include at least the following information: name of instrument, serial number and/or identification number of instrument, date of calibration, and calibration results. These logs shall be provided to the ACT Chief Scientist and maintained in a master calibration file as part of the QA/QC records.

a. Lab Experiment Quality Control Blanks. Blanks will be prepared weekly (at a minimum) during the laboratory BB3 and phytoplankton experiments.
b. Lab Experiment Quality Control duplicates. Collect duplicate samples weekly (at a minimum) during the laboratory BB3 and phytoplankton experiments.

7.4. Field Quality Control – Mooring and Profiling Deployments

Field quality control represents the total integrated program for assuring the reliability of measurement data. It consists of the daily field logs, quality control samples, and sample custody procedures.

7.4.1. Field Logs

Standard, uniform field logs should be maintained for all fieldwork. These logs should report name of staff conducting fieldwork, date (month, day, and year), operating status of all equipment, and manual readings of environmental conditions.

7.4.2. Field Quality Control Samples

To ensure that the reference sample collection and analysis procedures are properly controlled, field blanks and laboratory replicate samples will be taken once a week during the test period. These will be analyzed in the same manner as the collected reference samples and should comprise a minimum of 5% of the total samples collected and shipped.

a. Field Blank: Sample containers filled with distilled or deionized water are taken to the field and returned. This sample assesses contamination during transport and storage.
b. Sampler Device Blank: This sample is obtained by passing deionized water through a non-dedicated sampler, such as a portable pump, collection bottle, or rosette bottle. This blank is used to test contamination by a sampler.
c. Filtration Blank: Sample is collected by passing the deionized water through the field filtering apparatus to test the contamination by a filter and apparatus.
d. Field Duplicate: Two or more samples are collected simultaneously at a location to determine the variability associated with sample collection. This is to occur weekly at random during the test.

7.4.3 Sample Custody

All reference samples will be accompanied by the sample collection sheet and Chain-of-Custody (COC) form (see Appendix XX). The COC specifies time, date, sample location, unique sample number, requested analyses, sampler name, required turnaround time, time and date of transaction between field and laboratory staff, and name of receiving party at the laboratory. Proper labeling of sample bottles is critical. The COC is a mechanism by which a sample can be tracked through the various phases of the process: collection, shipping, receiving, logging, sample prep/extraction, analysis and final data QA/QC review.

7.4.4 Sample Handling

All collected reference samples at each test site will be handled in the same manner. Each reference sample should be dated and coded according to site and sample sequence. The actual sample container should be labeled with a number for identification. The reference sample number should be used in all laboratory records and COCs to identify the sample. Transfer of reference samples from field personnel to lab personnel is also recorded on the COC and records are maintained in the lab with the names and signature of persons leaving and receiving the custody. Samples stored for any period of time shall be routinely inspected by the TC to assure proper preservation and label integrity. The storage containers and storage devices (i.e. freezers and refrigerators) must be inspected routinely for proper operation and integrity. Results of all inspections shall be included in the sample records. All logs shall be duplicated weekly. The original shall be retained at the ACT Partner site and a copy shall be sent to the ACT Chief Scientist.

7.5. Audits

Independent of each Partner test facility QA activities, the ACT Chief Scientist will be responsible for ensuring that the following audits are conducted as part of this verification test at a minimum of three ACT Partner test sites. Audits shall be performed by Quality Assurance Specialists, who shall be independent of direct responsibility for performance of the verification test.

Performance Evaluation Audits – A performance evaluation audit will be conducted to assess the quality of the reference measurements made in this verification test. Run a known NIST-traceable standard (independent of the HPLC calibration standard) on the HPLC once during the test. This can be an independent standard or set of standards from a different vendor

or a different Lot# from the same vendor. Reference Instrument bias can then be calculated. This audit will be performed once during the verification test.

7.5.1. Technical Systems Audits

ACT's Quality Assurance Specialists will perform a TSA at least once during this verification test. The purpose of this audit is to ensure that the verification test is being performed in accordance with the test/QA plan, published reference methods, and any SOPs used by the Partner test facility. In this audit, the ACT Quality Assurance Specialists may review the reference methods used, compare actual test procedures to those specified or referenced in the test/QA plan, and review data acquisition and handling procedures. A TSA report will be prepared, including a statement of findings and the actions taken to address any adverse findings.

7.5.2. Data Quality Audits

ACT's Assurance Specialists will audit at least 10% of the verification data acquired in the verification test to determine if data have been collected in accordance to the test/QA plan with respect to compliance, correctness, consistency, and completeness the ACT Quality Assurance Specialists will trace the data from initial acquisition to final reporting.

7.5.3. Assessment Reports

Each assessment and audit will be documented, and assessment reports will include the following:

a. Identification of any adverse findings or potential problems,
b. Response to adverse findings or potential problems,
c. Possible recommendations for resolving problems,
d. Citation of any noteworthy practices that may be of use to others, and
e. Confirmation that solutions have been implemented and are effective.

7.6. Corrective Action

The ACT Chief Scientist, during the course of any assessment or audit, will identify to the ACT Technical Coordinators performing experimental activities any immediate corrective action that should be taken. If serious quality problems exist, the ACT Chief Scientist is authorized to stop work. Once the assessment report has been prepared, the ACT Chief Scientist will ensure that a response is provided for each adverse finding or potential problem and will implement any necessary follow-up corrective action. The ACT Quality Assurance Specialists will ensure that follow-up corrective action has been taken.

7.7. QA/QC Document Control

It is the responsibility of the ACT Chief Scientist to maintain QA/QC records, which shall include the following:

a. records of the disposition of samples and data.
b. records of calibration of instruments.
c. records of QA/QC activities, including audits and corrective actions.

8. Roles and Responsibilities

The verification test is coordinated and supervised by the ACT Chief Scientist and ACT Partner institution personnel. Staffs from the Partner institutions participate in this test by installing, maintaining, and operating the respective technologies throughout the test; operating the reference equipment, collecting the water samples, downloading the data from the instrument package, and informing the ACT Chief Scientist staff of any problems encountered. Manufacturer representatives shall train ACT Partner staffs in the use of their respective technologies and, at their discretion, observe the calibration, installation, maintenance, and operation of their respective technologies throughout the test. QA oversight is provided by the ACT Quality Managers. In addition to aiding the development of these protocols, the ACT Fluorometer Technical Advisory Committee will be consulted during the evaluation in the event problems occur, will assist in the analyses of results, and will review the final Verification Statement prior to release. Specific responsibilities are detailed below.

The ACT Chief Scientist has the overall responsibility for ensuring that the technical goals and schedule established for the verification test are met. The ACT Chief Scientist shall:

- Prepare the draft Test Protocols/QA Plan and Verification Statements.
- Revise the draft Test Protocols/QA Plan and Verification Statements in response to reviewers' comments.
- Coordinate distribution of the final Test Protocols/QA Plan and Verification Statements.
- Coordinate testing, measurement parameters, and schedules at each ACT Partner institution testing site.
- Ensure that all quality procedures specified in the test/QA plan are followed.
- Respond to any issues raised in assessment reports and audits, including instituting corrective action as necessary.
- Serve as the primary point of contact for manufacturers and ACT Partner Technical Coordinators.
- Ensure that confidentiality of proprietary manufacturer technology and information is maintained.

ACT Quality Managers for the verification test shall:

- Review the draft Test Protocols/QA Plan and Verification Statements.
- Conduct a technical systems audit (TSA) once during the verification test.
- Audit at least 10% of the verification data.
- Prepare and distribute an assessment report for each audit.
- Verify implementation of any necessary corrective action.
- Notify the ACT Chief Scientist if a stop work order should be issued if audits indicate that data quality is being compromised or if proper safety practices are not followed.
- Provide a summary of the audit activities and results for the verification reports.
- Review the draft verification reports and statements.
- Have overall responsibility for ensuring that the test/QA plan and ACT QMP are followed.
- Ensure that confidentiality of proprietary manufacturer technology and information is maintained.

ACT Technical Coordinators at each ACT Partner institution shall:

- Assist in developing the Test Protocols/QA Plan.
- Allow facility access to the manufacturers and ACT Headquarters representatives during the field test periods.
- Select a secure location for the tests.
- Install, maintain, and operate the fluorometer test systems at the test location at their respective institution.
- Perform sample collections and analyses as detailed in the test procedures section of the test/QA plan.
- One member of TC team will conduct 10% data audit as described in QA procedures. This will be done all data logs and electronically entered data.
- Provide all test data to the ACT Chief Scientist electronically, in mutually agreed upon format.
- Remove sensor systems and other related equipment from the test facility upon completing the verification test.
- Provide the ACT Chief Scientist and Quality Managers access to and /or copies of appropriate QA documentation of test equipment and procedures (e.g., SOPs, calibration data).
- Provide information regarding education and experience of each staff member involved in the verification.
- Assist in ACT's reporting of their respective test facility's QA/quality control results.
- Review portions of the draft Verification Statements to assure accurate descriptions of their respective test facility operations and to provide technical insight on verification results.

ACT West Coast Partner Institution, Moss Landing Marine Laboratories, shall:

- Perform reference measurements.
- Perform sample collections and analyses as detailed in the test procedures section of the Test Protocols.
- Provide the ACT Chief Scientist and Quality Managers access to and /or copies of appropriate QA documentation of test equipment and procedures (e.g., SOPs, calibration data).
- Provide information regarding education and experience of each staff member involved in the verification.
- Assist in ACT's reporting of their respective test facility's QA/quality control results.
- Review portions of the draft Verification Statements to assure accurate descriptions of their respective test facility operations and to provide technical insight on verification results.

Manufacturers shall:

- Review the draft test/QA plan and provide comments and recommendations.
- Approve the revised test/QA plan.
- Work with ACT to commit to a specific schedule for the verification test.
- Provide duplicate commercial-ready sensor systems for testing.
- Provide an on-site operator(s) to train ACT staff in the installation, operation, and maintenance of the sensor systems.
- Review and comment upon their respective draft Verification Statements.

Fluorometer Technical Advisory Committee shall:

- Assist in developing the Test Protocols/QA Plan.
- Approve the final Test Protocols/QA Plan.
- Provide specific advise during testing.
- Review and comment upon draft Verification Statements.
- Approve final Verification Statements.

9. Fluorometer Technical Advisory Committee

- Earle Buckley, North Carolina State University and ACT Advisor/QA Manager
- John Cullen, Dalhousie University
- Zbigniew Kolber, Monterey Bay Aquarium Research Institute
- Tom Johengen, ACT Partner and Cooperative Institute for Limnology & Ecosystems Research/University of Michigan
- Hugh MacIntyre, Dauphin Island Sea Lab / University of South Alabama
- Scott McLean, ACT Stakeholder and Satlantic
- Jan Newton, ACT Stakeholder and University of Washington
- Paul Pennington, NOAA Center for Coastal Environmental Health and Biomolecular Research and ACT QA Specialist
- Mary Jane Perry, University of Maine
- Collin Roesler, Bigelow Laboratory for Ocean Science
- Nick Welschmeyer, Moss Landing Marine Laboratories

10. Field Test Site Descriptions

Chesapeake Biological Laboratory Field Test Site –
The ACT Partner at Chesapeake Biological Laboratory (CBL), University of Maryland Center for Environmental Science, has established a Technology Verification Field Test Site on a fixed pier (Lat: 38°19.039 N, Lon: 76°27.065 W, with an average depth of 7 ft) at the mouth of the Patuxent River, a tributary of the Chesapeake Bay. The Chesapeake is a nutrient rich estuary with a watershed that encompasses portions of six states and the District of Columbia. Water temperatures at the testing location range from 0° to 35°C and salinities range from 5ppt to 20ppt depending on season, rainfall, wind, and other external factors.

Cooperative Institute of Limnology and Ecosystem Research Field Test Site –
The ACT Partner at the Cooperative Institute for Limnology and Ecosystems Research, University of Michigan, has established a Technology Verification Field Test Site on a fixed pier at the Lake Michigan Field Station of the NOAA Great Lakes Environmental Research Laboratory, in Muskegon, Michigan (43-13-40 N x 86-20-20W). The site provides direct access to Lake Michigan with water depth at the end of the pier averaging 3m. Lake temperatures range from 2 to 24°C on an annual basis. The field station operates a continuous real-time met station and fully equipped laboratories to process field samples.

Gulf of Maine Ocean Observing System Test Site –
The ACT Partner at the Gulf of Maine Ocean Observing System (GoMOOS) has established a Technology Verification Field Test Site at the University of Maine's, Darling Marine Center in Walpole, Maine. The Center occupies 170 acres of largely wooded property bordering 2 km of pristine water frontage on the Damariscotta River Estuary, and offers a secure and easy access to the estuary and maintains a pier and boating facility on site. Water sample analysis can be conducted during sensor evaluations in the laboratory facility near the pier. The Damariscotta River estuary is a tide dominated embayment approximately 5 km from the open waters of the Gulf of Maine. The site experiences a predominantly semi-diurnal tide with an approximate amplitude of 3m. Local marine environments include rocky shores, sandy beaches, mud flats, sea grass beds, and expansive sponge communities. The complexity of the Maine coastline allows for a wide range of exposure to waves and ice, further adding to the diversity of habitats. Sea temperatures range from 2 to 15 C° in the open ocean and from -2 to 20 C° in the upper reaches of the estuary. Salinity at the Center's dock ranges from 28 to 32 ppt.

Moss Landing Marine Laboratories Field Test Site –
The ACT Partner at Moss Landing Marine Laboratories (MLML) has established its Technology Verification Field Test Site at the MLML Small Boat Facilities (36.8041N, 121.7862W). This secure deployment site is located in Moss Landing Harbor on the junction of northern tributary of the Salinas River and Elkhorn Slough National Estuarine Reserve on the central coast of California. Instrumentation is deployed off a secure floating dock in waters with a tidal range of 2 meters and a maximum depth below the floating dock of 4 meters. It is an estuarine environment with a mean temperature of 12.858 ^{o}C (range: 11.287 to 15.767^{o}C) and a mean conductivity/salinity of 3.615 S m^{-1} / 30.577 PSU (range: 1.358 to 4.036 S m^{-1} and 10.851 to 32.942 PSU) at 1 meter depth.

Skidaway Institute of Oceanography Field Test Site –
The ACT Partner at the Skidaway Institute of Oceanography (SkIO) has established a Technology Verification Field Test Site on a floating dock adjacent to the Priest Landing Dock located on the eastern shore of Skidaway Island (Lat: 31° 57.768' N, Lon: 81° 00.705' W). Skidaway Island is sheltered from the Atlantic Ocean by a chain of barrier islands. The site experiences a semi-diurnal tide with a 2 m amplitude. The SkIO site is located within a typical subtropical estuary dominated by *Spartina alterniflora*. The Priest Landing dock is a large "T" shaped concrete structure that juts easterly into the north/south running Wilmington River. A minimum depth at test site is 14.28 ft. or 4.35m at MLW, water temperature range is 10 - 32°C and salinity range is 10 – 35 ppt.

University of Hawaii Field Test Site –
The University of Hawaii field site will be on the Kaneohe Bay Barrier Reef flat (157°48'W, 21°28.5') in waters ~2 m deep. Kaneohe Bay sits on the northeast, or windward, side of Oahu. The barrier reef acts as a physical divider separating coastal waters from the Kaneohe Bay lagoon and coastal ocean, as well as impeding the passage of surface wave energy into the bay interior. Significant wave heights at the study site are typically < 1 m with mean cross-reef currents only on the order of a few cm s-1. Both wave heights and cross-reef currents appear to be heavily modulated by the tides. Water temperatures at this site vary between 21 and 29°C

with highest values in summer. Tidal variations are typically less than 0.5 m and salinities are between 34.5 and 35.5 psu.

University of South Florida Field Test Site –
The ACT Partner at the University of South Florida (USF) has established its Technology Verification Field Site directly behind the College of Marine Science in Bayboro Harbor. Bayboro Harbor is located in the southwestern region of Tampa Bay, the largest Florida estuary and the second largest estuary in the eastern US. The deployment site (27° 45.612 N and 82° 38.003 W) is located at the end of a fixed dock, extending westward into the Harbor. This harbor protects two marinas, allowing for consistent and heavy recreational boat traffic. These waters have a summer temperature range from 27.5°C to 31.5°C with a mean of 29.5°C. The salinity varies from 20 psu to about 31 psu and is strongly dependent on rainfall amount. The site has a mean depth of 3.4 m and a mixed tidal range of about 1m. The dock sits on a soft bottom consisting mostly of unconsolidated sediments.

Protocols for Verifying the Performance of In Situ Chlorophyll Fluorometers

Amendment 1
April 25, 2005

4.1. Laboratory Tests

In Vivo Chlorophyll Detection, page 7 and 8

Instruments characterized for their BB3 fluorescence response will be evaluated for their response linearity and detection limit/range for in vivo chlorophyll fluorescence under standard reference conditions (32 ppt, 15^{o}C, dark). This will permit comparison of chlorophyll fluorescence in terms of the BB3 based RFU for assessment of instrument drift. The diatom *Thalassiosira pseudonana* Clone 3H (CCMP 1335) will be grown in a batch culture (75 µmol quanta m^{-2} s^{-1} PAR 40W fluorescent bulbs, continuous light at15^{o}C) with f/2 enriched seawater to mid-log phase (determined by cell counts), then propagated in semi-continuous culture at ca. 0.25 /d to maintain cell concentration at a level required for the dilution test. Instruments will be equilibrated as described above and allowed to collect 10 "blank" samples. Dark adapted (>1 h) 3H cells will be added and the measurements repeated. Chlorophyll samples will be taken as described above bracketing the measurement period (1st sample, 5th sample, l0th sample). 3H cells concentration will be sequentially increased to provide a range of target chlorophyll concentrations (e.g. 0, 100, 1000, 10000, 100000 cells / mL rapid proxy for chlorophyll added to the system).

To examine detection of multiple and taxonomically diverse species, a cyanobacteria (*Synechococcus* sp.) will also cultured as described above. In two separate test conditions, *Synechococcus* sp. will be added at either low concentrations (≤ 1,000 cells / mL) or high concentrations (approximately 100,000 cells / mL) to a bath containing an intermediate concentration of *Thalassiosira pseudonana*. The precise mixtures will be determined based on state and amounts of the two species available at the time of testing. Instruments measurements and water samples will be collected and processed as described above.

Protocols for Verifying the Performance of In Situ Chlorophyll Fluorometers

Amendment 2
May 12, 2005

4.1. Laboratory Tests

Page 7 – *Please note the addition of lower BB3 concentrations for testing in the following three sections.*

Response Linearity – For the linearity or stability tests, a mean and standard deviation of 5 instrument readings at 1-minute intervals for each test condition will be collected after the instruments are allowed at least 30 minutes to equilibrate. This instrument mean and STD will be compared to the mean and STD of 5 water samples BB3 samples collected at the same 1-minute intervals and quantified by absorbance spectroscopy. Test baths will filled with DI water (to provide a baseline or zero response factor), held in the dark. At least six concentrations of BB3 will be tested over the range of 0-15 µg / mL (e.g. 0, 0.001, 0.005, 0.01 0.05, 0.5, 1, 5 and 15 µg / mL) and temperature (4, 15 and 32 ^{o}C) will be varied to produce a matrix with 18 independent conditions for evaluating response linearity.

Detection Range – The experimental matrix above will enable determination of the linear detection ranges at each test temperature. Limit of detection will be computed as: (Mean + 3 S.D. of blank readings) and upper detection range will be determine as either the dye concentration causing saturation of instrument output or a greater than 50% decline in response factor. Only tests conducted in dark conditions will be used to determine detection limits of the instruments. These range estimates will then be independently tested at a reference temperature of 15^{o}C by monitoring instrument output over a low range of BB3 concentrations (e.g., 0.001, 0.005, 0.01 and 0.025 µg / mL) and high levels of BB3 (e.g., 0.5, 1, 5, 10, 15 and 20 µg / mL). The exact BB3 concentrations tested may vary depending on instrument response or gain settings. These tests will be subsequently repeated at 15^{o}C using RWT as the reference dye.

Light and Turbidity – Sensitivity to ambient light and water clarity will then be assessed by exposing the test instruments to high light (>200 umol/m/s PAR) using 500W halogen worklights and varying turbidity levels. Instruments will be placed in a bath at 15^{o}C with 0.1 µg / mL of BB3 and response factors, measured as described above, obtained in darkened conditions and under high irradiance (ca 200 µmol quanta /m^{2} / s, near sensor heads) conditions will be compared. Two different levels of turbidity (very low, set by the water source, low and high, 400 NTU max.) will then be produced by adding combinations of Formazin and colored dissolved organic matter (CDOM, filtered coffee will be used for simplicity). The response linearity test repeated under high light and in the dark. Turbidity test conditions will be quantified by a benchtop turbidity sensor in NTU. CDOM will be determined by absorbance spectroscopy on filtered samples (see below).

Protocols for Verifying the Performance of In Situ Chlorophyll Fluorometers

Amendment 3
May 23, 2005

4.3. Field Tests

Page 8.

Approximately 7 days prior to their particular field deployment, each test site will collect 10 independent water samples over a one-hour period at the location where field tests will occur (*the number of samples was increased from 5 to 10*).

Page 10.

Filtering - All chlorophyll *a* samples from each ACT Partner test site will be filtered on 2.5 cm Whatman GF/F membranes immediately after collection (<30 min). Sufficient volume of sample water will be filter to get visible color on filter surface and the precise volume filtered (400 mL maximum) for each sample recorded. All filtrations will be done in low light (i.e. not in front of window, away from sunlight), with low vacuum pressure (<5 in Hg). As soon as fluid runs through the filter, it will be removed, folded twice with the sample on the internal surface, wrapped in foil, placed in cryovials (one filter/vial), and immediately frozen by immersing into liquid N_2 (*please note the subtle changes to this set of steps*). Quick-freezing in liquid N_2 improves extraction efficiency and provides excellent long-term storage with no degradation (Bidigare et al. 2005, Analysis of pigments by high-performance chromatography. In R. Andersen (ed) Algal Culturing Technique, Academic Press).

Cleaning - Filtration apparatus and sample storage vessels will be cleaned between use by wiping with a clean cloth and copious rinses (>5) with DI water. Once per day (*changed from once per week*) filtration apparatus will be wiped, rinsed and soaked for 15 minutes in a 2% solution of Micro-90 or equivalent detergent, followed by a second round of copious rinsing with DI water.

Clarifications

(1) All weekly field blanks will be collected on the same day of the week.

(2) The labeling of samples will follow the following format:

Field Site, Sample Number, Replicate or Type of Sample

Field Site -
ME = University of Maine/GoMOOS; **CB** = Chesapeake Biological Laboratory; **SK** = Skidaway Institute of Oceanography; **SF** = University of South Florida, **ML** = Moss Landing Marine Laboratories; **GL** = Great Lakes / University of Michigan; **HI** = University of Hawaii.

Replicate or Sample Type -
A, **B**, **C** and **D** = chlorophyll replicates; **S** = TSS sample; **M** = CDOM samples

P preceding all other letters and numbers = a pre-test sample to characterize the field site phytoplankton community and to refine the extraction process (if required).

For example:
ML125B = the 125th water sample collection taken from Moss Landing Marine Laboratories, replicate filter "B" for chlorophyll
SK16S = the 16th water sample collection taken from the Skidaway Institute of Oceanography, filter for total suspended solids.
PHI3 = the 3rd water sample / filtration collected from University of Hawaii at Coconut Island prior to initiating the field tests.