PERFORMANCE VERIFICATION STATEMENT
For the Chelsea Technologies Group FastBallast Fluorometer

TECHNOLOGY TYPE:	Ballast water compliance tools
APPLICATION:	Shipboard analysis of ballast water
PARAMETERS EVALUATED:	Response linearity, accuracy, and precision
TYPE OF EVALUATION:	Laboratory and field performance verifications
DATE OF EVALUATION:	Testing conducted from March to July 2016
EVALUATION PERSONNEL:	M.R. First, S.C. Riley, S.H. Robbins-Wamsley, V. Molina, T. Johengen, H. Purcell, G.J. Smith, E. Reavie, K. Carney, C.S. Moser, E.N. Buckley M.N. Tamburri, and L.A. Drake

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BACKGROUND AND OBJECTIVES

In an effort to mitigate the risk of transporting aquatic nuisance species, the United States Coast Guard (USCG) has finalized a rule limiting the concentrations of organisms in ships' ballast water discharged into US ports (US Coast Guard 2012). The specified concentrations are nearly identical (with the exception of not including limits for *Vibrio cholerae* in zooplankton samples) to those in the International Maritime Organization's (IMO) convention (IMO 2004). Further, the limits are consistent with those in the US Environmental Protection Agency's Vessel General Permit (VGP)—regulations on a suite of vessel operations, including the discharge of ballast water (US EPA 2013). In order to meet these limits, most ships will use a ballast water management system (BWMS). These systems incorporate a variety of technologies (including filtration, UV radiation, electrolytic chlorination, and deoxygenation) to ensure that the discharge water meets the specifications.

Determining concentrations of living organisms can require extensive effort and sensitive equipment, especially for sparse populations. For example, direct counts of living organisms ≥10 and <50 μm according to the method stipulated in the US Environmental Technology Verification (ETV) Program Protocol for land-based testing of BWMS requires (1) labeling organisms within a sample with a set of vital fluorophores and (2) tallying the organisms via epifluorescence microscopy (EPA 2010; Steinberg et al. 2011). Direct counts of living organisms yield concentrations comparable to the numerical standard. While this rigorous, complex, and time-consuming analysis is appropriate for verification testing of BWMS, it is typically not feasible to perform this analysis during routine shipboard inspections. Rather, simple, hand-held, field instruments ("compliance tools")—with the ability to rapidly assess that the ballast water *clearly* exceeds the discharge limits—will be of much greater value to the ship owner, the BWMS vendor, and the compliance officer. Compliance tools should immediately produce results that are reliable indicators of the concentrations of living organisms within a regulated size class and predict whether a sample meets or exceeds the discharge standard.

New or refined compliance tools require carefully considered test protocols for evaluating and verifying their performance. The overall goal of this *technology verification* was to evaluate the performance of potential compliance tools designed to rapidly assess ballast water discharge. The outputs of the compliance tools were compared to the standard, validated approach (i.e. epifluorescence microscopy; EPA 2010) used to quantify organisms ≥10 and <50 μm in size during verification testing of BWMS. The objectives outlined below support this goal:

- In a series of laboratory trials to be conducted at the Naval Research Laboratory in Key West, FL (NRL), determine **linearity**, **precision** and **accuracy** of the compliance tool with samples of algal monocultures over a range of concentrations, including concentrations below, equal to, and above the IMO and US discharge standard.

- Evaluate the relationship between numerical concentrations of living organisms ≥10 and <50 µm and the accuracy and precision of the instrument using ambient organisms collected from natural waters at three various locations (Key West, Chesapeake Bay, and Lake Superior).

INSTRUMENT TECHNOLOGY TESTED

This report describes the test of the **Chelsea Technologies Group FastBallast**. The instrument employs variable fluorescence fluorometry, an approach that measures chlorophyll *a* fluorescence at variable illumination intensities and intervals. These measurements are used to estimate concentrations of living organisms within an aliquot of water. As photosynthetic algae are abundant in the ≥10 and <50 µm size class, the instruments may provide a reasonable determination that a sample meets the discharge limit of 10 living organisms mL^{-1} in the ≥10 and <50 µm size class.

Upon completion of sample analysis, FastBallast displays the estimated cell concentration (mL^{-1}) based upon the fluorescence measurements. Further details of the operation of the FastBallast are available in the test plan (**Appendix A**).

PERFORMANCE EVALUATION TEST PLAN

The test protocol for this performance verification was developed at a conference with NRL and the Alliance for Coastal Technologies (ACT) personnel, the participating instrument manufacturers, and a technical advisory committee. The verification of the instrument included both laboratory and field experiments: these tests are summarized briefly in this document and in detail in the test protocol. Experiments were designed to challenge the compliance tool by analyzing ranges of concentrations—spanning from zero to well above the discharge standard. Measurements reported by the instrument were compared to the results of the standard technique, described below. The critical comparison was the agreement on the disposition of the sample: if both the compliance tool and the microscope count indicate concentrations ≥10 mL^{-1}, the methods agree. Likewise, if both methods determine concentrations are <10 mL^{-1}, the methods agree.

Laboratory Experiments

Laboratory tests examined the agreement between cell concentrations measured via microscopy and the compliance tool using two cultured microalgae: *Tetraselmis marina* (cell dimensions: 9-15 µm) and *Prorocentrum micans* (25-50 µm). The organisms represented cell dimensions towards the extremes of the ≥10 and <50 µm size class. For the laboratory experiments with cultured algae, all living cells were counted, even though some individuals may have been slightly larger or smaller than the size limits. Samples with either *T. marina* or *P. micans* were prepared by diluting stock cultures with 0.22-µm filtered seawater (FSW) to yield concentrations of 0, 5, 10, 20, 50, and 100 mL^{-1}. Additionally, two samples were prepared to examine

interferences from (1) dissolved and particulate materials and (2) disinfection byproducts (DBP). These samples contained 10 mL^{-1} of either *T. marina* or *P. micans*.

Field Experiments

Instrument performance was also tested in field experiments using ambient water samples collected from three locations representing a range of water temperatures, salinities, and community compositions: The Naval Research Laboratory (NRL; latitude 24.58°N; Longitude: 81.79°W) in Key West, FL represented offshore, high salinity, waters (temperature: 21°C; salinity: 36 psu). The Great Ships Initiative (GSI) in Superior, WI (46.71°N; 92.05°W) represented the Great Lakes (4°C; 0 psu). The Smithsonian Environmental Research Center (SERC; 38.89°N; 76.54°W) in Edgewater, MD, located on the Chesapeake Bay, represented estuarine waters (29°C; 13 psu). Samples with a mixed assemblage of ambient organisms were prepared by either diluting or concentrating natural water from the location: dilution was performed by mixing the sample with FSW (or at GSI, 0.22-µm filtered *lake* water, FLW). Cells were concentrated by screening water through a sieve with mesh netting to retain organisms ≥10 µm. Following these procedures, four samples were generated with different target concentrations:

- 0 mL^{-1}, the 0.22-µm filtered water to be used as a control or blank for fluorescence,
- 5 – 20 mL^{-1}, representing concentrations near the discharge standard (DS),
- 30 – 50 mL^{-1}, representing concentrations above the DS, and
- ≥50 mL^{-1}, representing concentrations well above the DS.

Determining Concentrations of Microalgae by Epifluorescence Microscopy

Organisms ≥10 and <50 µm were quantified using the approach in the Environmental Technology Verification (ETV) Program protocol (EPA, 2010), namely, labeling organisms with a set of vital, fluorescing probes and manually counting fluorescent organisms via microscopy. This is the standard method used in land-based verification of ballast water management systems, and test participants designated this as the reference method for evaluating compliance tools. Fluorophores—chloromethylfluorescein diacetate (CMFDA) and fluorescein diacetate (FDA)—are added to a water sample. After a brief (10-min) incubation period, the sample is transferred into a gridded counting chamber, and a portion of the chamber is scanned for organisms moving, fluorescing, or both. Fluorescing organisms encountered were identified to general taxonomic group (e.g., dinoflagellates, diatoms, etc.) and manually tallied on a datasheet. At GSI, a validation study demonstrated that a single fluorophore (FDA) yielded equivalent counts of organisms as the dual set, so at this site, only FDA was used to label organisms. The detailed protocol for this approach is in **Appendix A**.

Measuring cell concentrations and exceedances using FastBallast

The instrument was contained in water-resistant case, and the instrument was configured to run in either flow-through or batch analysis mode (batch analysis mode was used for these experiments). Sample water was used to rinse the internal sample chamber, then, the sample was filled, the chamber closed, and the readings were collected by prompting the instrument to collect data. Upon completion of the analysis routine, the computer monitor displayed the cell concentration (mL^{-1}) and exceedance (≥ 10 mL^{-1}), which were manually recorded on a datasheet.

RESULTS

Linearity

The linear response of the FastBallast was measured by the change in reported concentration relative to the measured concentration of organisms ≥10 and <50 µm. Results of the laboratory trials are not available, as an instrument malfunction prevented data collection during the laboratory trials. Results of the field trials are shown in Figure 1. For field trials, linear regression was used to generate a line-of-best-fit describing the relationship between concentration and abundance. A linear relationship indicates the compliance tool's measurements will vary in proportion to the number of organisms in the sample. The strength of that relationship is measured by the coefficient of determination (R^2), a relative measurement (ranging from 0 to 1) that indicates how well the measurement conform to the line-of-best fit. Linear regression was performed on data from all trials for each organism or field site as well as the combined data set (from both organisms and all field sites). Results of linear regression analyses are shown in Table 1.

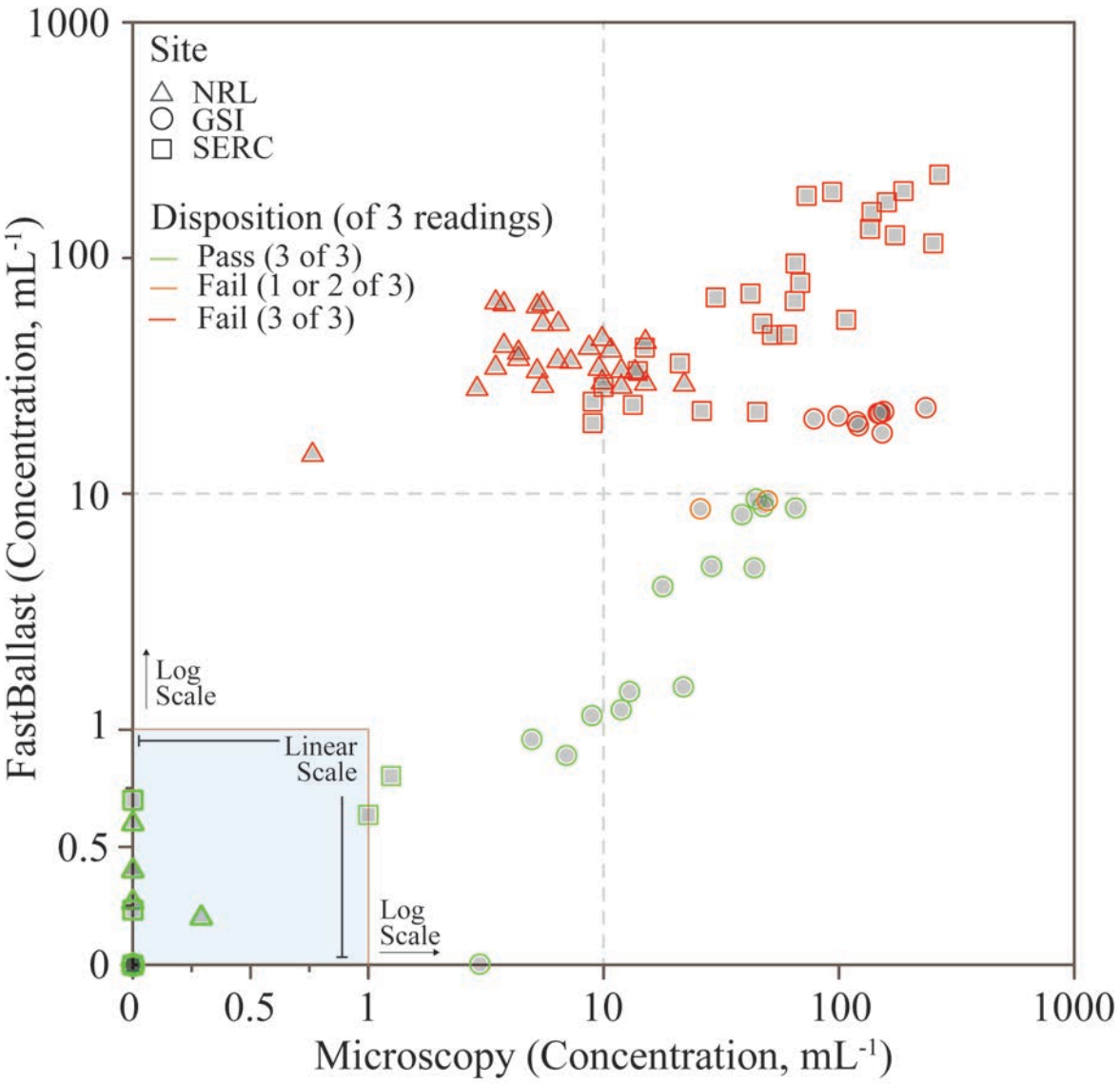

Figure 1. Results of the field experiments. Measurements from the FastBallast are compared to concentrations of ambient organisms ≥10 and <50 µm at the three test sites. Symbols mark the mean concentration. Symbol outlines display the number of repeated readings meeting (Pass) or exceeding (Fail) the discharge standard of <10 mL^{-1}. The figure inset has a linear scale. The rest of the figure displays data on a logarithmic scale.

Table 1. Results of linear regression analyses for both laboratory and field trials. Values indicate the adjusted (Adj.) R^2 value, the standard error (SE) of the estimates, *F*-values, slopes and y-intercepts (int.) of the relationship between cell concentrations measured by microscopy and FastBallast. Note: laboratory data is not available (N/A), as an instrument malfunction prevented analysis during the laboratory trials.

Data Set	Adj. R^2	R^2 SE	*F*-Value	Slope (±SE)	y-int. (±SE)	n
Laboratory Trials						
All organisms	N/A	N/A	N/A	N/A	N/A	N/A
T. marina	N/A	N/A	N/A	N/A	N/A	N/A
P. micans	N/A	N/A	N/A	N/A	N/A	N/A
Field Trials						
All Sites	0.356	37.7	$F_{1,105} = 57.9$	0.53 ± 0.07	16.10 ± 4.38	107
NRL	0.129	18.1	$F_{1,33} = 6.01$	1.41 ± 0.58	22.98 ± 4.66	35
GSI	0.867	3.14	$F_{1,34} = 228$	0.13 ± 0.01	0.69 ± 0.68	36
SERC	0.906	20.5	$F_{1,34} = 338$	1.06 ± 0.06	12.62 ± 0.06	36

All p-values for regressions <0.001

In the field trials, the linear relationships between concentrations of organisms ≥10 and <50 µm and measured by microscopy and by FastBallast were significant ($p<0.001$), with R^2 values highest for GSI and SERC ($R^2 = 0.87$ and 0.91, respectively; Table 1). The slope of the relationship between the two measurements of concentration was 1.06 ± 0.06 for SERC samples (Table 1).

Precision

Precision is a measure of the variation among repeated analyses. The precision of the instrument was determined by calculating the coefficient of variation (CV, %), a relative measure of the variation among replicate readings. CV is sensitive to small mean values (e.g., mean cell concentration <10 mL^{-1}): as mean approaches 0, CV approaches infinity. Because of this, the CV of mean values <10 mL^{-1} were reported, but only CV from samples ≥10 mL^{-1} were used to determine the range (Table 2). For field trials, the CV of three readings ranged from 2 to 31% (11% and 9%, mean and median CV, respectively, n = 22; Table 2).

Table 2. Mean, standard deviation (SD), and coefficient of variation (CV) of concentration measurements in laboratory trials (n = 3 for each sample). Black circles mark samples with concentration mean values ≥10 (no units); these values were used in the summary of the CV ranges reported in the text.

		Cell concentration (mL^{-1})		
Sample	**Trial ID**	**Mean ± SD**		**CV**
Control	NRL-1	0.5 + 0.1		17%
	NRL-2	0.3 + 0.1		23%
	NRL-3	13.1 + 1.2	●	9%
	GSI-1	0 + 0		173%
	GSI-2	0 + 0.1		173%
	GSI-3	0 + 0		Undefined
	SERC-1	0.4 + 0.4		103%
	SERC-2	0.1 + 0.1		173%
	SERC-3	0.2 + 0.2		100%
Near DS	NRL-1	56.7 + 4.7	●	8%
	NRL-2	33.1 + 6.4	●	19%
	NRL-3	63.4 + 2.4	●	4%
	GSI-1	1.4 + 0.1		7%
	GSI-2	0.9 + 0.3		27%
	GSI-3	0 + 0		Undefined
	SERC-1	23.1 + 1.8	●	8%
	SERC-2	24.1 + 2.2	●	9%
	SERC-3	36.9 + 1.9	●	5%
Above DS	NRL-1	39.4 + 1	●	2%
	NRL-2	34.9 + 7.1	●	20%
	NRL-3	39.3 + 5.7	●	14%
	GSI-1	8.9 + 0.2		3%
	GSI-2	8.7 + 0.8		10%
	GSI-3	4.6 + 0.3		7%
	SERC-1	49.8 + 1.1	●	2%
	SERC-2	63.9 + 2.4	●	4%
	SERC-3	79.8 + 9.3	●	12%
Well Above DS	NRL-1	34.8 + 4.4	●	13%
	NRL-2	34 + 10.1	●	30%
	NRL-3	32.3 + 10.1	●	31%
	GSI-1	20.6 + 1.6	●	8%
	GSI-2	21.9 + 1.8	●	8%
	GSI-3	20.1 + 2.4	●	12%
	SERC-1	124.6 + 3.2	●	3%
	SERC-2	177.5 + 8.8	●	5%
	SERC-3	197.4 + 17.1	●	9%

Accuracy

Accuracy of the instrument is a measure of the difference between a measurement and the actual or expected value, i.e. how good data are when compared with a recognized standard for measuring organisms ≥10 and <50 µm. (Note: from the Test Protocols "Accuracy is measured as the proportion of samples that correctly assess whether a sample meets the discharge standard"). For each sample read, the instrument reports whether the sample meets (*Pass*) or exceeds (*Fail*) the DS based upon cell concentration. A logistical regression analysis was used to determine the probability that the instrument correctly identifies the sample as *Pass* or *Fail* as cell concentrations diverge from the DS, whether below the DS (e.g., 0 to 9 mL) or above the *DS*. Concentrations were scaled so that values ≥10 mL^{-1} should be identified as *Fail*: effectively, 10 was subtracted from all measured concentrations prior to analysis. Results of the logistical regression analyses are shown in Table 3.

Table 3. Logistic regression results for the field trials. Note: laboratory data is not available (N/A), as an instrument malfunction prevented analysis during the laboratory trials.

		Constant (*C*)			Coefficient (*x*)			
		Value	**SE**	**p-Value**	**Value**	**SE**	**p-Value**	**n**
Laboratory Trials	Both organisms	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	T. marina	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	P. micans	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Field Trials	All Sites	0.038	0.221	0.863	0.028	0.009	0.003	108
	NRL*	28.1	35.4	0.428	2.87	3.57	0.421	36
	GSI*	-134	7305	0.985	2.15	118	0.985	36
	SERC*	20.74	1493	0.989	5.817	342	0.989	36

* Insignificant parameter fit (p>0.05)

To visualize the results of this analysis, the resulting values—the constant (*C*) and the coefficient (*x*)—were used to calculate the probability (ρ) of a High *Risk* (*H*) outcome across a range of cell concentrations (*P*):

EQ. 1

$$\boldsymbol{\rho(H) = \frac{1}{(1+e^{(-C+xP)})}}$$

Resulting $\rho(H)$ values across a range of cell concentrations are shown in Figure 3. As laboratory results were not available (due to an instrument malfunction), and as only one logistic regression analysis was significant (p<0.05), only one plot is shown.

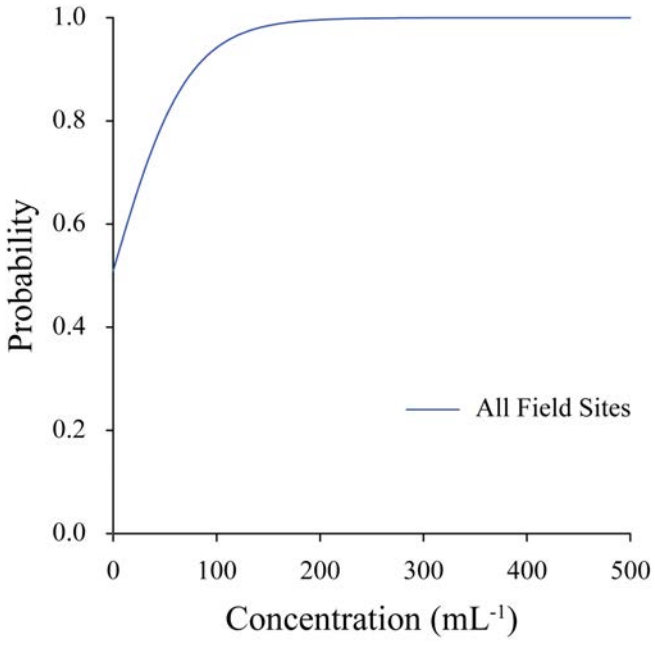

Figure 2. Probability of indicating a sample is High *Risk* based upon cell concentrations in field trials.

QUALITY MANAGEMENT

All technical activities conducted by ACT and NRL comply with their respective 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. Both organizations' QMS meet U.S. Environmental Protection Agency quality standards for environmental data collection, production, and use. The QMS also meets the requirements of General Requirements for the Competence of Testing and Calibration Laboratories (ISO/IEC 17025:2005[E]).

An effective assessment program is an integral part of a quality system. The ACT Quality Assurance (QA) Manager independently conducted six Technical Systems Audits (TSA, described below) and data quality assessments of all reference data sets for the evaluation.

Technical System Audits

A TSA is a thorough, systematic, on-site qualitative audit of sampling and measurement processes and procedures associated with a specific technology evaluation. The objectives of the

TSAs conducted during this evaluation were to assess and document the conformance of on-site testing procedures with the requirements of the Test Protocols and associated Standard Operating Procedures (SOPs).

The TSAs were conducted in accordance with the procedures described in 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 each audit and reviewed by the respective laboratory's personnel. The TSA assessed the respective laboratories' personnel, the test and analytical facilities, equipment maintenance and calibration procedures, sample collection, analytical activities, record keeping, and QC procedures. The audits were conducted for all field trials and laboratory trials.

During each audit, the auditor met with each person involved in testing and asked that person to describe the procedures. All procedures were observed, and logbooks, data forms, and other records were reviewed.

Key components of each audit included assessments of the following:

Quality Assurance/Quality Control:

- Adequacy of procedures and adherence to procedures
- Chain of command regarding description of assignments and specific duties

Sample System:

- Sample collection
- Analytical procedures
- Analytical equipment maintenance and calibration
- Documentation.

Data and Document Control:

- Chain of custody
- Validation and processing procedures
- Documentation

The findings of the TSA for the four field tests and two laboratory tests were positive. All of these tests were being implemented consistent with the Test Protocols and SOPs. Minor deviations were documented in laboratory records. None of the deviations had an effect on data quality for the evaluation Test Instruments. Failures were due to mechanical problems with the instrument. All phases of the implementation of the test reviewed during the TSAs were acceptable and performed in a manner consistent with ACT/NRL data quality goals. The overall quality assurance objectives of the test were met.

ACT and NRL 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 and NRL staff understands the need for QC, as shown in the conscientious development and implementation of a variety of QC procedures.

All samples and instrument measurements were collected, analyzed and cataloged 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.

Data Assessments

Data review was conducted to ensure that only sound data that are of known and documented quality and meet quality objectives were 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).

At the outset of the evaluation, data were verified and validated to evaluate whether data were generated according to the Test Protocols, satisfied acceptance criteria, and were appropriate for their intended use of evaluating the performance of the test instruments. Data verification evaluates the completeness, correctness, and consistency of data sets against the requirements specified in the Test Protocols, measurement quality objectives, and any other analytical process requirements contained in SOPs. The ACT QA Manager reviewed the reference (microscopy) data sets from all field and laboratory tests. Thirty-six (36) reference samples were counted for each field test (total 216 microscopy counts); fifty-six (56) reference samples were counted for each laboratory test (total 112 microscopy counts). The overall reference data set included 328 microscopy counts. The data review verified that the sampling and analysis protocols specified in the Test Protocols were followed, and that the ACT/NRL measurement and analytical systems performed in accordance with approved methods, based on the following criteria:

- The raw data records were complete, understandable, well-labeled, and traceable
- All data identified in the Test Protocols were collected
- QC criteria were achieved
- 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 data set. A representative set of approximately 10% of the reference 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. Validation of the referenced data set established:

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

- Required analytical methods were used

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

A Data Quality Assessment (DQA) is the third and final process of the overall data assessment. It is a scientific and statistical evaluation of validated data to determine if data are of the right type, quality, and quantity to support conclusions on the performance of the test instruments. The DQA determined that the evaluation's data quality objectives, described in the Test Protocols (Appendix A) were achieved.

REFERENCES

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The vendor's formal response letter is appended as a separate document.

APPENDIX A: TEST PLAN

Available for download at www.act-us.info/evaluations.

APPENDIX B: RAW DATA

Table 1. Summary of trials conducted.

Location	Trial Name	Trial Date	Trial Replicate
Naval Research Laboratory (NRL; Key West, FL)	NRL-1	3/2/2016	1 of 3
	NRL-2	3/3/2016	2 of 3
	NRL-3	3/4/2016	3 of 3
Laboratory Trial (LAB; Key West, FL)	LAB-1	3/5/2016	1 of 3
	LAB-2	3/6/2016	2 of 3
	LAB-3	3/7/2016	3 of 3
Great Ships Initiative (GSI; Superior, WI)	GSI-1	3/29/2016	1 of 3
	GSI-2	3/30/2016	2 of 3
	GSI-3	3/31/2016	3 of 3
Smithsonian Environmental Research Center (SERC; Edgewater, MD)	SERC-1	7/19/2016	1 of 3
	SERC-2	7/20/2016	2 of 3
	SERC-3	7/21/2016	3 of 3

Table 2. Concentrations of living organisms ≥10 and <50 µm in samples from field trials. Target concentrations were Control (0 mL^{-1}), near the discharge standard (DS, 5 – 20 mL^{-1}), above the DS (30 – 50 mL^{-1}), and well above the DS (>50 mL^{-1}).

Trial Number	Sample		Concentration (mL^{-1}) NRL	GSI	SERC
1 of 3	Control	A	0	5	0
		B	0	0	1
		C	0	0	1
	Near DS	A	6	22	45
		B	6	12	26
		C	3	13	9
	Above DS	A	7	48	60
		B	*	45	108
		C	10	26	52
	Well Above DS	A	22	79	173
		B	15	120	252
		C	15	100	136
2 of 3	Control	A	0	2	0
		B	0	3	2
		C	0	5	0
	Near DS	A	4	7	13
		B	6	9	9
		C	5	5	10
	Above DS	A	4	50	42
		B	3	39	30
		C	3	66	47
	Well Above DS	A	14	148	138
		B	12	151	94
		C	11	156	73
3 of 3	Control	A	0	4	0
		B	1	9	0
		C	0	4	4
	Near DS	A	6	15	15
		B	4	10	21
		C	5	11	14
	Above DS	A	6	29	65
		B	9	18	65
		C	4	44	69
	Well Above DS	A	12	236	267
		B	10	154	188
		C	10	122	160

Table 3. Concentrations of cultured organisms in samples from laboratory experiments. In two samples, the cultured organisms—*Tetraselmis marina* and *Prorocentrum micans*—were amended with dissolved and particulate materials or disinfection byproducts (DBP). Target concentrations ranged from 0 to 100 mL^{-1}. Note that laboratory tests were not performed for the FastBallast due to an instrument malfunction.

		Concentration (mL^{-1})	
Trial	**Sample**	***T. marina***	***P. micans***
LAB-1	0 mL^{-1}	0	0
	5 mL^{-1}	1	4
	10 mL^{-1}	2	11
	20 mL^{-1}	3	18
	50 mL^{-1}	18	46
	100 mL^{-1}	17	93
	10 mL^{-1} (Amended)	2	10
	10 mL^{-1} (DBP)	6	10
LAB-2	0 mL^{-1}	0	0
	5 mL^{-1}	1	5
	10 mL^{-1}	1	10
	20 mL^{-1}	7	13
	50 mL^{-1}	18	44
	100 mL^{-1}	55	89
	10 mL^{-1} (Amended)	3	6
	10 mL^{-1} (DBP)	4	10
LAB-3	0 mL^{-1}	5	0
	5 mL^{-1}	3	5
	10 mL^{-1}	5	8
	20 mL^{-1}	7	17
	50 mL^{-1}	16	58
	100 mL^{-1}	19	103
	10 mL^{-1} (Amended)	6	10
	10 mL^{-1} (DBP)	6	8

Table 4. FastBallast cell concentrations (mL^{-1}) of samples from field trials at NRL. Red symbols (●) indicate an exceedance of the discharge standard (DS).

			Replicate Reading				
Trial	**Sample**		**1 of 3**	**2 of 3**	**3 of 3**	**Mean**	**SD**
NRL-1	Control	A	0	0	0.2	0	0
		B	0.4	0.4	0.4	0.40	0.00
		C	0.7	0.4	0.7	0.60	0.17
	Near DS	A	46.2 ●	64.4 ●	47.1 ●	52.57	10.26
		B	37.5 ●	54.8 ●	65.3 ●	52.53	14.04
		C	71.4 ●	63.7 ●	59.8 ●	64.97	5.90
	Above DS	A	25.7 ●	52.1 ●	31.3 ●	36.37	13.91
		B	44.1 ●	43.6 ●	21.6 ●	36.43	12.85
		C	51.4 ●	22.3 ●	62.6 ●	45.43	20.80
	Well Above DS	A	25 ●	39.6 ●	22.6 ●	29.07	9.20
		B	32.3 ●	34.8 ●	20.9 ●	29.33	7.41
		C	39.3 ●	44.2 ●	49.1 ●	44.20	4.90
NRL-2	Control	A	0.2	0.5	0.5	0.40	0.17
		B	0	0	0	0	0
		C	0.3	0.3	0.2	0.27	0.06
	Near DS	A	60.3 ●	11.5 ●	40.3 ●	37.37	24.53
		B	27.9 ●	32.9 ●	25.3 ●	28.70	3.86
		C	33.3 ●	42.6 ●	23.5 ●	33.13	9.55
	Above DS	A	49.2 ●	46.8 ●	31.5 ●	42.50	9.60
		B	35.6 ●	41.3 ●	26.1 ●	34.33	7.68
		C	22.9 ●	36.4 ●	24.4 ●	27.90	7.40
	Well Above DS	A	30.8 ●	24.5 ●	43.4 ●	32.90	9.62
		B	16 ●	18.3 ●	51.1 ●	28.47	19.63
		C	34.6 ●	45.1 ●	42.1 ●	40.60	5.41
NRL-3	Control	A	10.4 ●	14.3 ●	12.3 ●	12.33	1.95
		B	16.1 ●	14.6 ●	13.2 ●	14.63	1.45
		C	12.5 ●	14 ●	10.1 ●	12.20	1.97
	Near DS	A	66.5 ●	64.4 ●	60.9 ●	63.93	2.83
		B	65.3 ●	50.8 ●	75 ●	63.70	12.18
		C	56.6 ●	68.9 ●	62.1 ●	62.53	6.16
	Above DS	A	31 ●	47.9 ●	31 ●	36.63	9.76
		B	38.7 ●	42.1 ●	44.2 ●	41.67	2.78
		C	35.6 ●	47.4 ●	36.2 ●	39.73	6.65
	Well Above DS	A	26.9 ●	49.5 ●	23.3 ●	33.23	14.20
		B	32.1 ●	36.6 ●	20.5 ●	29.73	8.31
		C	51 ●	32.2 ●	18.6 ●	33.93	16.27

Table 5. FastBallast concentrations (mL^{-1}) of samples from field trials at GSI. Red symbols (●) indicate a High (or *Fail*) *Risk* of exceeding the discharge standard (DS).

			Replicate Reading				
Trial	Sample		1 of 3	2 of 3	3 of 3	Mean	SD
GSI-1	Control	A	0.2	0	0	0.07	0.12
		B	0	0	0	0.00	0.00
		C	0	0	0	0.00	0.00
	Near DS	A	1.2	1.9	1.4	1.50	0.36
		B	1.1	1.3	1.2	1.20	0.10
		C	2.1	1	1.2	1.43	0.59
	Above DS	A	8.7	8.7	8.9	8.77	0.12
		B	8.2	9	11.1	9.43	1.50
		C	10.4 ●	8.3	7	8.57	1.72
	Well Above DS	A	22.6 ●	18.3 ●	21 ●	20.63	2.17
		B	20.7 ●	19.1 ●	20.3 ●	20.03	0.83
		C	24.2 ●	21.2 ●	18.2 ●	21.20	3.00
GSI-2	Control	A	0	0	0	0.00	0.00
		B	0.3	0	0	0.10	0.17
		C	0	0	0	0.00	0.00
	Near DS	A	0.9	0.8	0.6	0.77	0.15
		B	1.4	1	1	1.13	0.23
		C	1.3	0.9	0.5	0.90	0.40
	Above DS	A	10.2	7.8	9.8	9.27	1.29
		B	9.4	7.8	7.1	8.10	1.18
		C	9.3	8.6	8	8.63	0.65
	Well Above DS	A	19.1 ●	22.4 ●	23.9 ●	21.80	2.46
		B	21.7 ●	18.9 ●	24.8 ●	21.80	2.95
		C	22.1 ●	21.2 ●	23.2 ●	22.17	1.00
GSI-3	Control	A	0	0	0	0.00	0.00
		B	0	0	0	0.00	0.00
		C	0	0	0	0.00	0.00
	Near DS	A	0	0	0	0.00	0.00
		B	0	0	0	0.00	0.00
		C	0	0	0	0.00	0.00
	Above DS	A	5.1	5.2	4.3	4.87	0.49
		B	4.4	4.1	3.5	4.00	0.46
		C	4.7	4.9	4.8	4.80	0.10
	Well Above DS	A	25.3 ●	19.5 ●	24.2 ●	23.00	3.08
		B	20.6 ●	17.6 ●	15.7 ●	17.97	2.47
		C	20.9 ●	15.6 ●	21.4 ●	19.30	3.21

Table 6. FastBallast concentrations (mL^{-1}) of samples from field trials at SERC. Red symbols (●) indicate a High (or *Fail*) *Risk* of exceeding the discharge standard (DS).

			Replicate Reading				
Trial	Sample		1 of 3	2 of 3	3 of 3	Mean	SD
SERC-1	Control	A	0	0	0	0.00	0.00
		B	0.7	0.4	0.2	0.43	0.25
		C	1.6	0.3	0	0.63	0.85
	Near DS	A	27 ●	22.8 ●	17 ●	22.27	5.02
		B	19.7 ●	21.9 ●	25.9 ●	22.50	3.14
		C	24 ●	29.4 ●	20.4 ●	24.60	4.53
	Above DS	A	44 ●	48.4 ●	49.7 ●	47.37	2.99
		B	49.8 ●	57.9 ●	56.6 ●	54.77	4.35
		C	53.6 ●	41.4 ●	46.8 ●	47.27	6.11
	Well Above DS	A	139.1 ●	109.3 ●	127.2 ●	125.20	15.00
		B	116.3 ●	116.4 ●	114 ●	115.57	1.36
		C	118.9 ●	138.3 ●	142.2 ●	133.13	12.48
SERC-2	Control	A	0.7	0	0	0.23	0.40
		B	0	0	0	0.00	0.00
		C	0	0	0	0.00	0.00
	Near DS	A	30.8 ●	23.3 ●	17.5 ●	23.87	6.67
		B	16 ●	19 ●	24.9 ●	19.97	4.53
		C	32.9 ●	27.5 ●	24.8 ●	28.40	4.12
	Above DS	A	79.1 ●	66.5 ●	67 ●	70.87	7.13
		B	62.4 ●	73.9 ●	68.1 ●	68.13	5.75
		C	54.4 ●	55.4 ●	48.5 ●	52.77	3.73
	Well Above DS	A	156.5 ●	163.5 ●	151.5 ●	157.17	6.03
		B	204.9 ●	188.4 ●	180.7 ●	191.33	12.36
		C	192.5 ●	188.4 ●	170.8 ●	183.90	11.53
SERC-3	Control	A	1.4	0	0.7	0.70	0.70
		B	0	0	0	0.00	0.00
		C	0	0	0	0.00	0.00
	Near DS	A	41.8 ●	50 ●	33.5 ●	41.77	8.25
		B	29.9 ●	40.3 ●	37.1 ●	35.77	5.33
		C	32.5 ●	25 ●	42.1 ●	33.20	8.57
	Above DS	A	78 ●	61.4 ●	58.2 ●	65.87	10.63
		B	97.9 ●	92.1 ●	95.1 ●	95.03	2.90
		C	70.9 ●	109.6 ●	55.2 ●	78.57	28.00
	Well Above DS	A	223 ●	193 ●	263 ●	226.33	35.12
		B	220.2 ●	196.3 ●	162 ●	192.83	29.25
		C	207.7 ●	179.1 ●	132 ●	172.93	38.22

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The study described within this report compares estimates of phytoplankton cell density provided by the FastBallast active chlorophyll fluorometer and the EPA (2010) epifluorescence microscope method.

Active chlorophyll fluorometry, in general, is widely seen as an enabling technology for the on-board interrogation of ballast water discharge due to its ability to detect viable phytoplankton cells. All active fluorometer systems are able to measure variable chlorophyll fluorescence (Fv) which is highly correlated with photochemical activity. Because photochemical activity provides the energy required for cell growth and division, Fv is generally seen as a reliable indicator of cell vitality.

The FastBallast system incorporates a 'level 1' test which effectively assumes that each phytoplankton cell within the test sample emits a set level of Fv (Fv $cell^{-1}$). In reality, Fv $cell^{-1}$ is roughly proportional to cell volume and, for phytoplankton cells within the 10 – 50 µm range, can vary by orders of magnitude. It follows that selection of an intermediate Fv $cell^{-1}$ value could result in both false positives (if the sample is dominated by large cells) or false negatives (if the sample is dominated by small cells). For this reason, the level 1 test is only implemented when the total Fv signal from the sample is higher than anticipated from 10 or more large cells mL^{-1}. In all other situations (including all of the tests within this study) the FastBallast runs a 'level 2' test which estimates cell density from the distribution of a large number of Fv values around the mean. Within this particular study, 200 to 240 measurements of Fv were acquired at 1 Hz within each level 2 test. These acquisitions were made from a 0.5 mL interrogated volume within a 20 mL sample. The sample was stirred at a rate that was slow enough to provide minimal exchange during each acquisition but fast enough to ensure that the entire sample was interrogated over the test period. From theory, this procedure should generate a normal distribution with a standard deviation equal to the square root of the mean (approximating a Poisson distribution). A non-iterative algorithm incorporated within FastBallast makes use of this distribution to provide a real time estimate of cell density. Importantly, the cell density reported by a level 2 test is independent of Fv $cell^{-1}$ and actually allows for simple calculation of the mean Fv $cell^{-1}$ value within the sample.

Extensive modelling and lab-based experiments have verified that this approach works well for cell densities from 2 to more than 1000 cells mL^{-1}, in most situations. One notable exception is where there are a few cells within the sample with a very high Fv $cell^{-1}$ and a larger number of cells with a very low Fv $cell^{-1}$. In this situation, the cells with a high Fv $cell^{-1}$ can effectively hide the cells with a low Fv $cell^{-1}$.

At the other end of the scale, there are no known circumstances where the algorithm incorporated within FastBallast will generate an overestimate of cell density. Having said that, it has to be acknowledged that a false positive result could be generated by a significant proportion of cells within a sample being less than 10 µm in the smallest dimension.

Mean Fv $cell^{-1}$ values from each sample are not included within this report, but are easily derived from the test data (which includes cell density and mean Fv values). Some discussion of Fv $cell^{-1}$ values is included within this response.

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The data set from the NRL site includes a number of points that failed the FastBallast test, but were assessed as having fewer than 10 cells mL^{-1} during microscope-based assessment. These data could indicate that a proportion of the cells detected by FastBallast were excluded from the microscope-based count because they were below 10 µm in the smallest dimension. However, it is worth noting that the mean Fv $cell^{-1}$ values from the NRL samples were actually quite high (consistent with a spherical cell of approximately 23 µm diameter) and it may be that a significant proportion of the cells that emitted Fv did not emit sufficient FDA-dependent fluorescence to be counted as living cells.

The data set from GSI shows a high R^2 value for the plot of microscope-based cell counts against FastBallast estimates of cell density (0.8664 when forced through the origin) but a very low slope for this relationship (0.14). The mean Fv $cell^{-1}$ value from the GSI tests was relatively high (consistent with a spherical cell of approximately 33 µm diameter). Consequently, it seems likely that FastBallast has underestimated cell density because a low number of large cells has effectively hidden a larger number of small cells, as discussed above.

The data set from SERC show an excellent correlation between microscope-based cell counts and FastBallast estimates of cell density. The mean Fv $cell^{-1}$ was slightly lower than from NRL, being consistent with a spherical cell of approximately 21 µm diameter.

To some extent, these test results provide a useful illustration of the strengths of the FastBallast system and areas that have required improvement. The NRL data draw attention to the possible inclusion of cells that are below 10 µm in the smallest dimension within the cell count. To address this issue, we are assessing the best way of including a low Fv $cell^{-1}$ limit within the PASS / FAIL decision (a software-based modification). At the other end of the scale, the GSI data illustrate the possible underestimation of cell density within samples that include cells with widely differing Fv $cell^{-1}$ values. We are in the process of implementing a filtration step (15 µm mesh) which will only be triggered in situations where the reported Fv $cell^{-1}$ from the initial test is high and reported cell density is low. A second test will then be run on the filtrate to test for the presence of smaller cells.

Disappointingly, none of the field test samples would seem to have been dominated by cells at either end of the 10 – 50 µm range, which is where the level 2 test shows the greatest advantage over a level 1 test. Unfortunately, technical issues with the FastBallast units submitted for the SERC tests prevented completion of the lab tests, which did incorporate small cells. Although these issues were resolved for the NRL and GSI tests, the lab tests were not repeated at these sites.

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