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Independent Laboratory Auditing Protocol

For Facilities Performing Type Approval Testing of Ballast Water Management Systems

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N O T I C E

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16. Abstract (MAXIMUM 200 WORDS) This report describes an audit protocol (including a set of auditing procedures and checklists) for biological efficacy testing performed at land-based, Ballast Water Management System (BWMS) test facilities under the authority of a U.S. Coast Guard-approved Independent Laboratory. The development of these audit procedures occurred through visiting four test facilities between November 2017 and June 2019. During these visits, the project team identified living organism concentrations in treated discharge water as a key performance metric for BWMS. The report documents audit goals, general findings, and provides recommended auditing procedures. The auditing protocol is segmented into six parts, which includes procedures that occur prior to, during, and following the onsite audit. The auditing procedures include a set of checklists and worksheets, which guide auditors during the documentation review and the onsite visit. Implementing these auditing procedures could improve testing towards the goal of strengthening the type approval process.		
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EXECUTIVE SUMMARY

Modern shipping's use of ballast water has facilitated the spread of aquatic nuisance species (ANS), as organisms suspended in ballast water are loaded in one location and discharged elsewhere. Following the introduction of ballast-water-mediated ANS, regional, national, and international actions resulted, limiting the allowable concentrations of organisms in ballast discharges. To meet the current discharge standard, most commercial vessels will install a "ballast water management system" (BWMS), which is a relatively new class of marine equipment. Before new systems are installed on vessels, BWMS manufacturers must obtain type approval (TA). The USCG Marine Safety Center (MSC) is responsible for reviewing, accepting, and approving TA applications. Under this process, BWMS manufacturers are required to work with a USCG-accepted Independent Laboratory (IL), which will conduct, *inter alia*, land-based biological efficacy (BE) testing. The IL typically relies on test facilities (TFs) to conduct land-based testing.

This project developed an auditing protocol for USCG-approved ILs, specifically for TFs performing land-based BE testing. The requirements for land-based TA testing of BWMSs have been driven by recent ballast water discharge regulations and recognition that auditors may not be familiar with the complexities of BE testing. To conform with test requirements cited within the regulations, individual test facilities have had to develop their own purpose-built test apparatus and associated standard operating procedures. Given the relatively recent development of BWMS test processes, the methodology for conducting an audit of land-based TFs is not yet established.

This report describes an audit protocol (including a set of procedures and checklists used to compile and document objective evidence) for BE testing performed at land-based TFs. The development of this protocol took place during four TF site visits between November 2017 and June 2019. The study approach first identified unique challenges associated with BE testing, where concentrations of living organisms in the ballast discharge is a key performance metric for BWMSs. Next, the project team examined goals for auditing an IL with an established quality management system. This report documents the general findings from the site visits. Based on the research, the project recommends an auditing protocol that includes six procedures.

1. Initial preparation and document review (pre-audit).
2. IL & TF management meetings and staff interviews (onsite).
3. Challenge water preparation (onsite).
4. Uptake and discharge operations (onsite).
5. Sample processing and analysis (onsite).
6. Audit team wrap-up meeting and report generation (post-audit).

The report describes the roles and responsibilities of the Lead Auditor and auditing team as well as the requirements for each procedure. Checklists (and supporting worksheets) are started during the pre-audit document review and finished during the onsite audit. In addition, this report provides a list of recommended changes to the BE testing process that can strengthen the TA process.

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF TABLES

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LIST OF ACRONYMS, ABBREVIATIONS, AND SYMBOLS

ANS	Aquatic Nuisance Species
APW	Alkaline Peptone Water
BWMS	Ballast Water Management Systems (plural, BWMSs)
BE	Biological Efficacy
CFR	Code of Federal Regulations
CFU	Colony Forming Unit
CUC	Control Union Certifications
CUW	Control Union Water
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
DMSO	Dimethyl sulfoxide
DNV	Det Norske Veritas
DQI	Data Quality Indicator
EPA	Environmental Protection Agency
ETV	Environmental Technology Verification
FITC	Fluorescein isothiocyanate
GWRC	Great Waters Research Collaborative
IMO	International Maritime Organization
IL	Independent Laboratory (plural, ILs)
ISO	International Organization for Standardization
KIOST	Korean Institute of Ocean Science & Technology
KOMERI	Korean Marine Equipment Research Institute
KR	Korean Register
MDL	Method Detection Limit
MEA-NL	Marine Eco-Analytics (Netherlands)
MEPC	Marine Environment Protection Committee
MM	Mineral Matter
MSC	Maritime Safety Center
NIVA	Norwegian Institute for Water Research
NRL	United States Naval Research Laboratory
OFI	Opportunities for Improvement
POC	Particulate Organic Carbon
POM	Particulate Organic Matter
PSU	Practical Salinity Units
QMS	Quality Management System
UV	Ultraviolet
SCADA	Supervisory Control and Data Acquisition
STO	Standard Test Organism
TA	Type Approval
TCBS	Thiosulfate-citrate-bile salts-sucrose
TF	Test Facility (plural, TFs)
TQAP	Test Quality Assurance Plan

LIST OF ACRONYMS, ABBREVIATIONS, AND SYMBOLS

TSS	Total Suspended Solids
UN	United Nations
USCG	United States Coast Guard
USDA	United States Department Of Agriculture
VIDA	Vessel Incidental Discharge Act
WET	Whole Effluent Toxicity
WMR	Wageningen Marine Research

1 BACKGROUND

Ballast water, which is used by ships to control draft, trim, stability, or stress, has facilitated the spread of aquatic nuisance species (ANS), as water is loaded in one place and discharged elsewhere. Following the introduction of ballast-water-mediated ANS, regional, national, and international actions resulted. At the international level, the United Nations' (UN) International Maritime Organization (IMO) adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments (IMO 2004, hereafter the *Convention*), which entered into force on September 8, 2017. It established a discharge standard for ships' ballast water to reduce the transport and delivery of potential ANS. In the United States several legislative and executive actions governing ballast water discharges resulted in regulations promulgated by the U.S. Coast Guard (USCG) and Environmental Protection Agency (EPA) (e.g., U.S. National Archives and Records Administration 2012; EPA 2013). The purpose of the IMO and U.S. actions—to greatly limit the discharge of living organisms—will be carried out using essentially the same discharge standard, which considers organisms in two size classes: organisms ≥50 µm (nominally zooplankton) and organisms ≥10 µm and <50 µm (nominally protists). The discharges standard also limits concentrations of "indicator microbes" (Table 1).

Table 1. Ballast water discharge limits. Limits are similar between IMO and USCG, except that IMO considers 'viable' (rather than 'living' organisms). IMO also sets a limit of toxigenic *Vibrio cholerae* based upon zooplankton biomass (<1 cfu* per 1g wet weight zooplankton).

Organism Category	Limit
Living Organisms ≥50 µm in Minimum Dimension	<10 per m^3
Living Organisms ≥10 µm and <50 µm in Minimum Dimension	<10 per mL
Toxigenic *Vibrio cholerae* (Serotypes O1 and O139)	<1 cfu per 100 mL
Escherichia coli	<250 cfu per 100 mL
Intestinal Enterococci	<100 cfu per 100 mL

*cfu = colony forming unit

1.1 Ballast Water Management Systems

To meet the current discharge standard, most commercial vessels will install a "ballast water management system" (BWMS). In this case, ballast water may be treated upon intake, upon discharge, by in-tank dosing or treating, or using some combination of these approaches. Of the different BWMS available or in development, most treat water using a combination of physical separation (usually filtration) followed by a disinfection step (typically either electrochlorination or ultraviolet [UV] radiation). These approaches are widely used in the (non-maritime) water treatment industry (Drake 2017b). While land-based water treatment and its associated technologies have evolved over many decades in response to a succession of regulations, the ballast water industry is relatively nascent.

1.2 Type Approval Testing of Ballast Water Management Systems

Before BWMS are installed on vessels, BWMS manufacturers must obtain type approval (TA) granted by a flag state (i.e., a country in which vessels are registered). The USCG Marine Safety Center (MSC) is responsible for reviewing, accepting, and approving TA applications on behalf of the U.S. flag. Under this process, BWMS manufacturers are required to work with a USCG-accepted Independent Laboratory (IL), which will conduct land-based, shipboard, and environmental testing, in addition to safety and design reviews. The IL will then submit the test reports and other required documentation in support of the manufacturer's TA application to MSC. The IL typically relies on *sub-laboratories* to conduct testing, analyses, or both. Sub-laboratories performing land-based *biological efficacy* (BE) testing are also known as *test facilities* (TFs). Currently, the USCG has accepted five ILs actively engaged in TA testing (Table 2). U.S. regulations also require that the testing organizations employ a quality management system (QMS) in accordance with ISO/IEC 17025:2005 (hereafter, ISO 17025).

Table 2. USCG accepted Independent Laboratories (ILs) actively engaged in TA testing following 46 CFR §162.060. The sub-laboratories performing biological efficacy testing are listed for each IL.

IL	Abbreviation	Sub-laboratory (or laboratories)
Control Union Certifications BV	CUC	Control Union Water (CUW) Wageningen Marine Research (WMR)[1] Great Waters Research Collaborative (GWRC)
Det Norske Veritas GL AS	DNV	DHI-Denmark BallastTech NIVA AS Golden Bear Research Center Korean Marine Equipment Research Institute (KOMERI) Marine Eco-Analytics (MEA-NL)
Korean Register of Shipping	KR	DHI-Denmark KOMERI
Lloyd's Register EMEA	LR	BallastTech NIVA AS DHI Denmark KOMERI Marine Eco-Analytics (MEA-NL) BallastTech NIVA AS
Korean Institute of Ocean Science and Technology	KIOST	KIOST

Updated list: https://cgmix.uscg.mil/EQLabs/EQLabsSearch.aspx; accessed 30-Jul-2019
[1]Formerly known as IMARES

The U.S. requirements for the TA application and verification testing are codified in the U.S. Code of Federal Regulations (CFR; 46 CFR §162.060). Specifically, land-based testing should proceed according to the Environmental Technology Verification (ETV) Program Generic Protocol for the Verification of Ballast Water Treatment Technology (ETV Protocol; EPA 2010). The ETV Protocol was developed—with the input of stakeholders and technical experts—under the ETV Program in a joint effort between the USCG and the EPA.

1.3 Project Goals

The goal of this project was to develop an auditing protocol for USCG-approved ILs, specifically for TFs performing land-based, BE testing. Other required laboratory testing—such as environmental testing (to verify that the components can withstand shipboard conditions, such as vibration) and Whole Effluent Toxicology (WET) testing—have been used for many years, and there are numerous established standard procedures (as well as knowledgeable auditors familiar with these procedures). Contract laboratories conducting these familiar tests typically operate under an accredited QMS such as ISO 17025, which require audited processes for recordkeeping, training, equipment maintenance, sample handling, quantification of measurement uncertainty, etc. Audits of these facilities are conducted by personnel trained in the standard methods, required records and types of measurements conducted. Auditors are expected to be familiar with test elements, technical capabilities, and supporting organization infrastructure.

In contrast, the requirements for land-based testing of BWMS have been driven by recent ballast water discharge regulations. To conform with test requirements cited within the regulations, individual test facilities needed to develop their own purpose-built test apparatus and associated standard operating procedures (SOPs), where the measurement uncertainties are unique to each facility. Given the recent development of BWMS test processes, the methodology for conducting an audit of many aspects of land-based test facilities is not yet established. This project developed the approach for auditing of these facilities.

This report first identifies unique challenges associated with BE testing, as concentrations of living organisms in the ballast discharge is a key performance metric for BWMS. The research team conducted four site visits to TFs representing four ILs. Site visits occurred from November 2017 until June 2019. The site visits are described in separate trip reports, written for a limited distribution, as the reports contained business-sensitive information. This report describes the *general findings* from the site visits.

Based on the research and site visits, the report recommends audit procedures. Each procedure has a bulleted list of instructions and actions. Audit tasks include the completion of several checklists, annexed to this document in Appendix A. Finally, from the site visits, discussion with TF and IL personnel, and document review, the report notes several items in the ETV Protocol that should be reconsidered or clarified.

This report focuses on the BE testing performed at land-based test facilities. Through document reviews and site visits, the project team addressed the topics described in *Experimental Design* chapter of the ETV Protocol (Chapter 5, EPA 2010[1]). The project team also considered other topics relevant to testing, such as: communication between the IL and sub-laboratory, testing independence, as well as responsibilities of the

[1] Note: future references to the ETV Protocol include the chapter or section number but omit the citation.

vendor, the TF, and the IL (ETV, Chapter 2). The following topics are beyond the scope of this report: compliance with requirements for determining acceptability of testing, operation and maintenance testing, communications with the USCG, and reporting verification results (i.e., ETV Chapters 3, 4, and 6).

Note that this report addresses TA testing as currently required in the USCG Final Rule (46 CFR §160.060); **it does not consider changes that may result from the Vessel Incidental Discharge Act (VIDA).**

2 TESTING CHALLENGES

The key challenges for evaluating BWMS originate from an unusual test *measurand*: mixed assemblages of living plankton, representing a spectrum of phenotypes, trophic strategies, and life stages. Organisms considered include single-celled bacteria, protists, microinvertebrates, larvae, and their resting stages (e.g., cysts and spores). In contrast, traditional bioassays typically track the response of a single, well-characterized species to a treatment. While the targeted community may include familiar taxa—as well as standard test organisms (STO) added to augment test water concentrations—the overall behavior of any single population in a mixed community is difficult to predict. Further, the community is dynamic: populations of bacteria and microalgae are capable of rapid growth, but populations are also subject to loss through predation (Calbet and Landry 2004) and viral lysis (Wommack and Colwell 2000). Certain populations may be sensitive to changes in their local environment (e.g., temperature and salinity), so that even modest manipulations may lead to mortality. These characteristics lead to the following challenges, described below.

2.1 Challenge: Lack of a Reference Standard

Analytical approaches demonstrate their accuracy with measurements of a known quantity, or a *reference standard*. The reference standard may be certified by a laboratory with knowledge—buttressed by empirical data—of the degradation rate (and thus, its shelf life) and measurement tolerances. Such a standard is not possible for a mixed assemblage of ambient plankton. Organisms concentrations change over hours (certainly days; e.g., Kim et al. 2011), so measuring the same reference standard over time would not yield a known or stable value to use as a baseline for comparison or a data quality indicator.

2.2 Challenge: Short Analytical Time Window

Samples generated from tank fill and drain operations must be analyzed soon after collection, as the biological assays distinguish between living and dead organisms. In contrast to other bioassays, the living plankton cannot be chemically preserved and stored for later analysis. Organisms, if living at the time of collection, would be sensitive to "bottle effects", which describe the suite of rapid changes in containment vessels that result in changes to the community (relative to the source community; e.g., Ferguson et al. 1989). Bottle effects include, e.g., depletion of dissolved oxygen and temperature changes. Effectively, analysis must occur onsite or nearby, so the personnel performing the analysis must begin their work promptly and continue their analyses as samples arrive throughout the day.

2.3 Challenge: Representative Sampling

Type approval testing seeks to examine BWMS under challenging conditions, at operational tempos reflective of those used on a ship (i.e., high volumetric flow rates). The ETV Protocol requires at least 200

m^3 volumes for both the control and treatment tanks. Tanks are filled at ≥200 m^3 h^{-1}, and sample water is collected continuously throughout the tank fill or drain operation. These engineering requirements pose additional challenges, such as ensuring representative sampling. Samples must be collected from the ballast piping through probes designed to extract water near the centerline of the pipe, while preventing damage to the sampled organisms due to shear stresses or impact with the sample collection apparatus. The ETV Protocol requirement for "isokinetic sampling" considers the diameters and flow velocities within the main pipe and sample probe. The pipe diameters for a single system are fixed, but changing flow velocities could shift the sample flow rate outside of the target range. The recommended sampling configurations are well defined and validated (Wier et al. 2015; 2017), but the challenges of maintaining the target sampling rate and of collecting and processing large volumes require substantial effort to monitor and control test parameters.

3 AUDITING GOALS

3.1 Verify Adherence to the USCG Testing Requirements

Testing requirements are defined in the Title 46 of the Code of Federal Regulations (CFR); key requirements are summarized below (Figure 1). The ETV Protocol, referenced in 46 CFR §162.060-5, contains the technical requirements for TA testing. Within the ETV Protocol, the main technical requirements are in Chapter 5, the source of the majority of elements listed within the audit checklists (Appendix A). An audit of a land-based TF will verify all the listed technical requirements are met.

3.2 Quality Management Systems (QMS)

In order to be accepted as an IL, laboratories must have an acceptable QMS; per 46 CFR §162.060-36 this must be in accordance with ISO 17025. This requirement extends down to the organization performing the testing, typically a sub-laboratory who may be affiliated with one or more ILs. The ISO 17025 QMS approach includes separate *Management* and *Technical Processes*; the former includes the organizational infrastructure needed to support business and technical operations, while the latter identifies requirements associated with the technical nature of the measurement and includes corroborating documentation.

Early on, the project team convened a meeting and invited several subject matter experts associated with QMS accreditation and BWMS field assessment to discuss and develop an audit plan that could be used to audit ILs (Drake 2017a). The resulting discussion provided details of common steps during an audit by an accreditation organization. During an audit, records, procedures, and process improvements are audited and may be observed. An auditor may also choose to perform a *Vertical Audit* that follows all processes used in the preparation of an outcome, such as a laboratory measurement. A vertical audit includes, e.g., the purchase of reagents, the training of the technician, examination of chain of custody for the sample under measurement, and calibration records of equipment used in the measurement. In any audit, an *Audit Checklist* is necessary in order to maximize the benefit of time spent on site. In addition, selected documentation can be reviewed prior to travel on site. In large scale testing—such as that used in BWMS testing—the installation locations of sensors, the procedures used for test preparations and analyses, and even the types of valves may impact the measurement and performance of the system under test. Here, the test setup, the sampling process, and the analytical methods are all factors in determination of measurement uncertainty. A draft agenda for the audit is provided so that auditors and onsite personnel can determine the

best days and times to conduct staff interviews, tour field or laboratory facilities, and observe key operations.

Meeting participants discussed QMS audit approaches, considerations associated with BWMS testing, and the desire to develop an audit procedure specific to IL performing such testing. Following these discussions, several key outcomes were identified:

- Measurement uncertainty is a primary consideration.
- Every test facility should have a dedicated quality manager.
- Multiple data quality indicators should be used.
- The onus should be on test facilities to demonstrate their procedures and provide documentation and evidence.
- Two-day audits should be completed with at least one biologist and one engineer (*note: the project later determined three-day audits were ideal for land-based TFs*).
- Test the hypothesis that most (if not all) laboratories have accreditation to ISO 17025, and so others can be relied upon to audit the scope of accreditation; thus, the audit can focus on processes *not* stipulated in ISO 17025, e.g., ballast water sampling.
- Having a quality system in accordance with ISO 17025 requires that sub-laboratories generate a statement on the validity of non-standard methods; most methods specific to testing a BWMS will be unique to the laboratory (especially those related to control and treatment tracks, representative sampling, measurement detection limits and uncertainty). Examining these technical aspects will likely provide the most insight into the degree of conformance with USCG regulations and requirements.

The above outcomes were incorporated into the approach in the audit research visits to each sub-laboratory, and the approach was revised after each visit. Common to all visits was the request and review of QMS documentation associated with USCG type approval testing directly from the laboratory. A visit was scheduled to occur during type approval tests and the associated test plan was also requested. Based on the review and completeness of the supplied documentation, certain operational processes were identified for observation, and questions on SOPs, practices, or assumptions requiring validation were noted for discussion onsite.

3.3 Harmonization among Multiple Test Facilities

As noted above, several TFs conduct TA testing for BWMS: They may differ in their approaches when implementing the requirements of the ETV Protocol. The challenge water—its chemical constituents, the natural assemblages of ambient organisms, and the effects of additives and STOs on the natural organisms—will *certainly* vary among TFs. Regardless of these differences, the network of TFs should demonstrate that a test outcome, i.e., whether a BWMS meets or exceeds the discharge limits, *does not* vary among the TFs. This issue was addressed in a project that tested one BWMS at two locations (Drake et al. 2012). Among other findings, the authors highlighted the importance of auditing, including pre-test internal audits, IL audits, and external technical systems audits, as described herein. The external auditor can improve harmonization by recommending opportunities for improvement, but TFs can also participating in information-sharing networks. These are described briefly below.

3.3.1 Opportunities for Improvement and Best Practices

Periodic audits will identify deficiencies but will also identify Opportunities for Improvement (OFI), which include, e.g., recommendations to simplify test methods, reduce analytical uncertainty, or improve laboratory practices. For example, an OFI may suggest an efficient approach for cleaning the piping system between trials or an optimal approach for positioning sensors. Likely, the OFI is a recommendation based upon the methods and procedures developed at another TF (and observed during an audit). Some of these *Best Practices* are proprietary, so cross-site sharing is not appropriate. However, TFs in the network of USCG-approved ILs participate in a forum where sharing best practices is encouraged—the Global TestNet.

3.3.2 Information Exchange among TFs (Global TestNet)

Global TestNet is an assembly of representatives from TFs. The name for this group originated at a GloBallast meeting, and in the Memorandum of Understanding, the portmanteau was defined as "Global Ballast Water Testing Organizations Network" (Linders 2012). Global TestNet is a forum for communications among TFs. Members agreed to communicate by correspondence and at annual meetings, and to "*discuss and share methodologies, analytical procedures and protocols used to support certification testing, and provide insight and lessons learned, to help improve the overall quality and efficiency of management technology testing"* (https://www.globaltestnet.org/Home; accessed 7/15/2019). Additionally, members recognize the importance of standardization among the TFs and may participate in cross-training and intercalibration.

3.4 Demonstration of Quality and Integrity

Audits demonstrate quality and integrity of the testing procedure to various stakeholders: BWMS manufacturers, vendors, regulatory authorities, shipowners, shipbuilders, and the communities affected by and concerned with bioinvasions. Testing for TA must assure stakeholders that the BWMS is capable of meeting the U.S. discharge limits (33 CFR §151.2030) under suitably challenging conditions. Periodic audits help to eliminate any perception that certain TF may be more or less rigorous than others, and that results across TFs are comparable. Noting that laboratories accredited to ISO 17025 will have both internal and external audits of their QMS, the particulars of the IL audit process here focuses on the ability of the laboratory to have suitable quality systems that address the requirements of the ETV. Audits are intended to assess conformity to the requirements of a standard, in this project, requirements are specified by 46 CFR §159.010 and 46 CFR §162.060, which in turn cite the ETV Protocol and ISO 17025. These are illustrated by refining the focus of the audit protocol to reflect the criteria listed below (Figure 1).

Independent Laboratories (ILs) must:

1. Perform testing as a regular part of their business
2. Possess materials and personnel necessary to inspect the test equipment (including "apparatus, facilities, personnel, and calibrated instruments")
3. Not be owned or controlled by the BWMS manufacturer, the vendor, or supplier
4. Not depend upon the Coast Guard to remain in business
5. Not advertise tested equipment

46 CFR §159.010-3 Independent Laboratory: Standards for Acceptance

ILs must:

1. Conduct a **readiness evaluation** to determine the acceptability of the BWMS for testing and the BWMS meets all environmental and safety regulations. Through the readiness evaluation, verify the tested BWMS is a complete system, intended for sale.
2. Prepare a written Test Plan following §162.060-24.
3. Conduct evaluations and report results according to the requirements of §162.060-34

46 CFR §162.060-42 Responsibilities for Independent Laboratories (ILs)

Test plans must:

1. Include an examination of the manufacturer's requirements for installation, calibration, operation and maintenance.
2. Include an accounting of environmental, health, and safety issues (including the disposal requirements of treated ballast water.
3. Conduct land-based tests in accordance with the ETV Protocol or obtain USCG approval for planned deviations in accordance with §162.060-10(b)(1).
4. Incorporate a Quality Assurance and Quality Control Program in accordance with ISO IEC 17025 as per §162.060-36

46 CFR §162.060-24 Test Plan Requirements

Land-based Testing:

1. Will determine whether the BWMS under test meets the discharge standard and validate the operating and maintenance parameters that are presented by the manufacturer.
2. Will follow the test plan requirements of the ETV Protocol. This includes "*five consecutive, valid, and successful replicate test cycles,"* following protocols detailed in the Operation, Maintenance, and Safety Manual.
3. Will include the following elements of each valid test cycle: ≥200 m^3 h^{-1} pumping rate, and ≥200 m^3 of water pumped to each the control and treatment tanks, and holding water for ≥24 hours.
4. Will test the BWMS in conditions (salinity ranges) for which it will be approved, and if the BWMS is downscaled from a larger size, the scaled unit must meet the minimum treatment rate and guidance for scale factors in §162.060-26(f)1.

46 CFR §162.060-26 Land-based Testing Requirements

Figure 1. Regulatory requirements relevant to the land-based TA testing considered in this project.

4 GENERAL FINDINGS OF THE SITE VISITS

The report briefly outlines several findings based upon 1) documentation review, 2) communications with representatives from several ILs, and 3) site visits to four test facilities. As mentioned above, the specific findings include business-sensitive information, and as the site visits were not formal audits, the project limited the distribution of specific findings to U.S. Government employees and contractors. The following general findings represented all or most (i.e., three of four) of the ILs test facilities.

The project team's observations only relate to documents provided to us by the test facilities and operations discussed or observed. For this project, the audit research focused primarily on technical processes associated with land-based test operations, challenge waters, sample collection, sample analysis, and associated data quality procedures. In particular, the TF may have an SOP covering a particular procedure or a validation study and report, but the document was not transmitted to the reviewers. In several cases, documents not transmitted initially were provided when specifically requested.

4.1 Test Management and Oversight

All TFs demonstrated effective test management and appeared to conform to descriptions of roles and authorities in the Quality Management Plan. Testing teams and laboratory teams, in most cases, had shared personnel, although some personnel would only work on either the testing in the field or analysis in the laboratory, but not both. During observations of onsite procedures, project staff observed that the teams communicated well, typically face-to-face, but also through hand-held radio and test documents (i.e., datasheets and logs). Project staff observed pre-test meetings to discuss test specifics prior to tests (ETV §7.1). The overall project managers (or TF Directors) were physically present for portions of the testing, but they were not necessarily engaged with the testing or analytical operations. Nevertheless, the project managers were nearby and appeared to keep in close communication with their staff. In some cases, personnel performing testing or analyses also served as Quality Officers. While onsite, personnel alerted the project team to potential safety issues and required wearing of personnel protective equipment (ETV, Chapter 9).

4.1.1 Opportunities for Improvement

Overall, the project found test management and oversight to follow the guidelines of the ETV Protocol and to be appropriate to assure proper execution of the testing. The project team notes that when personnel share multiple roles, their management and reporting duties should be clearly defined. An example would occur when a test operator or laboratory analyst may report to their area lead, but when serving as a Quality Officer, may report to the test facility director.

4.2 Evaluating Standard Test Organisms (STOs)

Three TFs used STOs to augment natural populations of organisms in their challenge water. The fourth TF did not use STOs; rather, they filter and concentrate the ambient population of organisms at their location and supplement the challenge water to ensure it meets the criteria thresholds. This TF's documentation indicated they could supplement organisms with cultured organisms. Other TFs concentrate ambient organisms *in addition* to augmenting with STOs. The ETV Protocol addresses requirements for evaluating biological efficacy using STOs. In particular, the TF should "conduct sufficient experimentation and

provide evidence indicating a broad resistance to treatments as outlined by Anderson, et al. (2008)," (ETV §3.6).

4.2.1 Opportunities for Improvement

TFs are required to perform validation experiments only when using organisms not listed specifically in the ETV (ETV §3.6). However, the ETV requires validation studies for both injection of organisms and additives (ETV §5.3.3) as well as organism mortality (ETV §5.4.6.4). **It is critical that STOs be evaluated in this manner, but the project also recommends that concentrated ambient organisms be evaluated to determine their survivability during injection**. (Note that this can – and probably should – be done in conjunction with a validation study comparing treatment and control tracks.) Concentrating organisms potentially raises their abundance to above the carrying capacity of their environment: oxygen demand increases, prey are depleted, and organism encounter rate increases, so that the rate of predation within a size class increases. These stressors may cause an increased sensitivity to treatment.

4.3 Test Water Sources

The requirements for source waters are best stated in the description of challenge water in the ETV Protocol:

> Natural water of less than 1 [Practicable Salinity Units] PSU will be used for fresh water conditions, while natural seawater will be used for marine conditions. Testing at multiple salinities at a given TF should only be conducted if there are natural water sources with the differing salinities (e.g., fresh and brackish waters). Artificial modification of the salinity of the waters should be used only if it can be demonstrated that the concentrations, diversity and condition of organism populations required in Section 5.2.2 will not be impacted by adjustment of the salinity. (ETV §5.2.1.1)

All four TFs conduct testing at all three salinities described in the ETV Protocol. In order to obtain all three water types, TFs:

- Transported water via ground, e.g., lorry (or multiple lorries).
- Transported water via ship.
- Used natural groundwater as a freshwater source.
- Added brine to increase salinity.
- Added dechlorinated municipal water to decrease salinity.

The manipulations of salinity (but also the addition of concentrated organisms and other materials) must not impact the diversity and condition of organisms. Changes in the organism community in the control tank effectively demonstrate the rate of survival of the different organism populations.

4.3.1 Opportunities for Improvement

The project team recommends the TFs produce a comprehensive study of the organism dynamics in the control tank, highlighting changes between the uptake and discharge samples. Organism dynamics would include changes in concentrations due to growth and reproduction or loss through predation and other forms of mortality. The report should include estimates of the concentrations of major taxa. Ideally this would be compared to dynamics in the treatment track without any treatment. This dataset should be updated as data become available and summarized periodically (annually).

4.4 Challenge Water Amendments

TFs augmented Dissolved Organic Matter (DOM), Particulate Organic Matter (POM), and Mineral Matter (MM) by adding:

- Lignin sulfonate, sodium citrate, humic material, or glucose for DOM.
- Cornstarch and commercially available dried algae for POM.
- Clay minerals (kaolin) for MM.

Several TFs used the dual addition of sodium citrate and lignin sulfonate to achieve a target optical clarity of the test water: sodium citrate is optically transparent upon dissolution, but lignin sulfonate is opaque, so balancing the contribution of the two additives will yield a specific UV absorbance. Laboratory-based validation at some TFs demonstrated the impact of these compounds on both UV absorbance and total residual oxidant (TRO) consumption (TF internal data). The choice of additives also will influence the biological community, particularly by stimulating bacterial growth and oxygen demand. These effects drive changes in organisms in larger size classes.

4.4.1 Opportunities for Improvement

Ideally, validation studies will compare challenge water augmented with additives to control challenge water. Both control and treatment will have the organism concentrations to meet challenge water criteria, supplementing organisms as would be performed in an actual test. Critically, the dissolved oxygen and organism concentrations (including bacteria) are monitored throughout the hold time and upon discharge. If TFs select additives to meet requirements of the BWMS (such as UV transmissivity), the justification for the selection, which may reflect the BWMS limitations, should be clearly communicated.

4.5 Test Facility Design and Infrastructure

The project team found TFs to be, in general, well equipped to conduct testing. In most cases, TFs prepared challenge water in a source tank prior to the experiment (one TF amended the water during the fill operation). All TFs complied with the in-line sampling requirements and used diaphragm valves to control sample flow. All TFs conducted fill operations sequentially, rather than splitting the flow of a single into parallel treatment and control lines (e.g., ETV §5.3.2.1), and all performed cleaning of tanks and piping to prevent cross-contamination between test runs.

4.5.1 Opportunities for Improvement

While test facilities met the requirement of the ETV Protocol, most TFs would benefit from the expanded use of a Supervisory Control and Data Acquisition (SCADA) system. Such a system is recommended, but not required (ETV §5.3.4). Nevertheless, a SCADA system simplifies test operations and data logging. This is especially helpful when logging flow rates, both in the main line and in the sample lines. In addition to the validation studies specifically recommended in the ETV, TFs should also validate their cleaning protocol to assure it is adequate. This follows from the recommendation that there is no source of contamination from previous use of the system (ETV §5.3.1).

4.6 Sampling Methodology

All TFs used similar approaches for in-line, representative sampling. Uptake samples were typically collected via periodic, discrete samples during, e.g., at the first, middle, and last third of the tank fill. All

TFs used plankton nets suspended in tanks or barrels to concentrate organisms ≥50 µm during discharge sampling. Often, three tanks and plankton nets were used for the first, middle, and last third of the tank fill. Sample volumes were generally sufficient to yield an appropriate method detection limit (MDL, e.g., MDL = 2 m^{-3} for determining concentrations <10 m^{-3}).

4.6.1 Opportunities for Improvement

The ETV Protocol requires that "…biological samples will be collected on a time-integrated basis such that a composite sample of the entire period of uptake or discharge is acquired" (ETV §5.3.2.7). Discharge sampling meets this procedure, especially when the concentrated samples from the beginning, middle, and last thirds of the discharge are pooled into a single volume. Collecting several discrete, small volume samples during the uptake may be appropriate, but the project recommends TFs harmonize their sampling procedures with the requirements of the ETV. For example, this could be accomplished by taking multiple discrete samples, pooling those samples, and subsampling the pooled sample.

4.7 Analysis of Organisms ≥50 µm

Test facilities generally showed compliance with ETV Protocol and its approach for analysis of organisms ≥50 µm. In one case, the vital label ***Neutral Red*** was used to assist with detecting and counting organisms. Its use to indicate vitality is not in compliance with the ETV method. The approach was used to supplement the required vitality (based upon movement), and it is not likely that Neutral Red interferes with required approach. Another TF indicated that they discontinued the use of Neutral Red to conform with the ETV protocol but were interested in performing a validation and, in turn, submitting a formal request for its use as an alternative method.

In most instances, there were multiple analysts independently enumerating organisms ≥50 µm. Interviews with analysts showed they were well-trained and experienced in microscopy and general plankton taxonomy. Photomicrographs were archived for possible future taxonomy and sizing needs. The ETV protocol allows for direct counts of living organisms at low concentrations (such as treated discharge) but recommends counting dead organisms first, fixing the organisms, then counting the plate again for the total count of organisms. Most of the TFs complied with this recommendation. In one instance, the period of observation of a non-moving organism following touching was less than the 10 seconds suggested in the ETV Protocol. At least one TF would periodically have an identical sub-sample counted by multiple analysts as a Data Quality Indicator (DQI).

4.7.1 Opportunities for Improvement

TFs should follow the ETV as written. In cases where methods deviated (even slightly) from the ETV,the project team recommends that TF perform side-by-side validation studies to determine whether the modified method generates concentrations estimates similar to the required method. TFs should incorporate DQIs to verify counting accuracy. The ETV method recommends a spike recovery approach, but other approaches may be appropriate (see ETV §5.4 for a complete description). TF should have multiple analysts to perform microscope counts of organisms ≥50 µm, especially for treated discharge samples, where large volumes must be analyzed within hours of collection. Analysts should demonstrate consensus in their analyses, primarily by practice analyses and periodic checks of an unknown sample.

4.8 Analysis of Organisms ≥10 and <50 µm

All TFs complied with the ETV Method for counting organisms ≥10 and <50 µm. In one case, the fluorophore solutions were prepared with acetone rather than dimethyl sulfoxide (DMSO), which in this instance, was shown to produce a greater fluorescence yield.

4.8.1 Opportunities for Improvement

In general, TFs complied with the ETV requirements and validated methodological deviations; no specific OFIs were identified. However, all microscopy-based analyses would improve with additional data quality indicators (as described below in Section 6.3). TFs performed a Most Probable Number (MPN)-based assay for International Maritime Organization (IMO) testing, so it is likely that TFs would be able to compare results of this assay to the ETV Method.

4.9 Analysis of Bacteria

Bacterial analysis include the following:

- Heterotrophic, culture, aerobic bacteria (hereafter, heterotrophic plate counts) as challenge water requires ≥1000 ml^{-1}.
- The indicator bacteria *E. Coli* and enterococci to verify discharges meet limits.
- Toxigenic *Vibrio cholerae* (serotypes O1 and O139), also to verify discharges meet limits.

The ETV Protocol requires standard microbiological techniques, such as proper equipment (biological safety cabinet, autoclave, personnel protective equipment), and all TFs were properly equipped. Personnel performing bacterial analyses wore proper safety equipment and performed work in dedicated areas, with proper equipment and supplies. All TFs used the IDEXX Colilert and Enterolert kits (IDEXX Laboratories; Westbrook, ME) to quantify *E. coli* and enterococcus, respectively. The ETV Protocol allows for this approach, and the TFs followed manufacturers recommended protocols for sample manipulation, incubation, and analysis. When possible,the project team observed the inoculation of the IDEXX Colilert and Enterolert media, sealing of the incubation chambers, and, on the following day, analysis.

All TFs, however, used approaches for quantifying toxigenic *V. cholerae* that differed from the ETV Protocol. The ETV Protocol requires a colony dot-blot hybridization targeting the *ctxA* gene (ETV §5.4.6.7) and incubation with 2.5% yeast extract and naladixic acid. While the TFs adopted standard approaches (e.g., Huq et al. 2012; ISO 21872), they did not incorporate the elements required in the ETV protocol. Typically, the TFs followed approaches outlined in the reference cited in the section (Huq et al. 2012), which listed multiple approaches for sample processing and analysis.

The TFs used the approach recommended by Huq et al. (2012) to selectively culture *V. cholerae*, namely, concentrating 100 mL on a filter, then incubating the filter in liquid media (alkaline peptone water, APW).

Following an overnight incubation, a "loopful" of bacteria are pulled from the pellicle of the culture media, i.e. the fluid below the culture surface, which is the strata containing microaerophilic bacteria. As a secondary stage, Huq et al. (2012) describe spreading the loop onto the surface of *Vibrio*-selective agar media (Thiosulfate Citrate Bile Salts [TCBS]). Analysts identify potential *Vibrio cholerae* based upon colony morphology, selecting suspect colonies for down-stream analysis to confirm the bacteria identity.

TFs differed in their approaches for positively identifying colonies, but they used well-accepted approaches (including those described in Huq et al., 2012) such as probing for specific genes or metabolic assays.

The alternative approaches likely have little impact on test quality, and likely, the TF approaches are better suited for unambiguous identification of toxigenic *V. cholerae* than the ETV approach, as the TFs methods are similar to those adopted as standard methods and used by public health agencies (e.g., ISO 21872, USDA 1992, etc.): metabolic assays or gene amplification are sensitive, specific, and—relative to the colony-dot plot assay quantified by epifluorescence microscopy—easy to perform, non-subjective, and definitive.

4.9.1 Opportunities for Improvement

TFs may have approval to use alternative methods for toxigenic *V. cholerae*, and if so, their approaches for bacteria are appropriate. Deviations from the ETV Protocol must be approved by the appropriate mechanism (i.e., 46 CFR §162.060-10(b)1). In the case of toxigenic *V. cholerae*, the ETV Protocol's required approach should be revised or clarified to include the approaches used by the TFs (see Section 6.4 below).

5 AUDITING PROTOCOL

Based on the research, this project has developed a proposed auditing protocol for IL sub-laboratories performing land-based BWMS testing. Here, an official audit would have the authority of the offices responsible for the certification of ILs and acceptance of their test evaluations[2]. As in accreditation audits, formal roles are identified. The *Lead Auditor* represents the USCG in communications with the IL, the TF, and others, and understands the responsibilities undertaken by the IL. The Lead Auditor also coordinates personnel on the audit team, which ideally will include technical experts well-versed in the ETV Protocol, BE testing, test and evaluation of marine equipment, or other relevant experience. The audit team may include individuals who participate only in document review, but onsite auditors should have performed a thorough review of the IL documentation and identified particular items to be observed and audited during the onsite visit.

The Lead Auditor (or designee) should be the only *Point of Contact* for arranging the audit, and all communications should be archived, e.g., in email records, notes of telephone communications, or summaries of in-person communications. This allows others to review the communications, verifying the accuracy and completeness of Lead Auditor's statements. The record also assures that communications would not be misinterpreted as official advice, guidance, or instructions from the USCG. The project team proposes a six part auditing protocol:

1. Initial preparation and document review (pre-audit)
2. IL & TF management meetings and staff interviews (onsite)
3. Challenge water preparation (onsite)
4. Uptake and discharge operations (onsite)
5. Sample processing and analysis (onsite)
6. Audit team wrap-up meeting and report generation (post-audit)

[2] Office of Design and Engineering (46 CFR §162.060-40), Marine Safety Center (46 CFR §162.060-40), *inter al.*

Figure 2 is a flowchart that shows the major parts of the audit, a brief description of the tasks, and the supporting documents for the tasks.

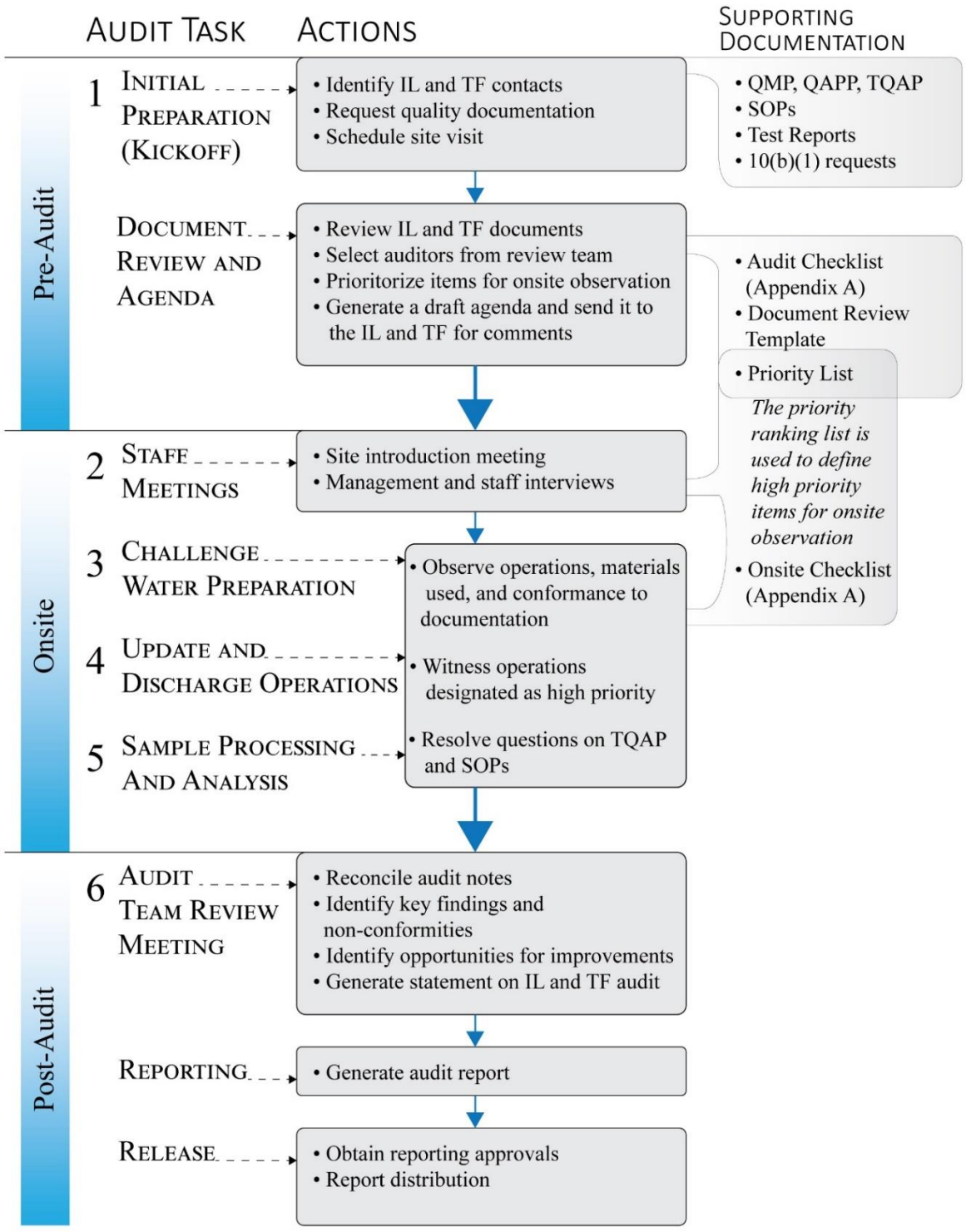

Figure 2. Flow diagram of the auditing procedure.

In support of the documentation and site audit of TF, several checklists itemize requirements in the USCG regulations, the ETV Protocol, and ISO 17025 (Table 3; Checklists are in Appendix A). Each part will have checklists with criteria corresponding to these requirements, and for each item, auditors will document the *objective evidence* to assess whether the TF is ***conforming***[3] or ***not conforming*** to the criteria. Checklists are completed throughout the various stages of the audit: each item may have information relevant to the pre-audit document review, the onsite audit, and the post-audit deliberations (Figure 3).

Table 3. Auditing checklists and references to the ETV Protocol.

Checklist (Appendix A)	ETV Section(s)
A.1 Quality Management System (QMS) Checklist*	§7, §8, and Appendix A*
A.2 Challenge Water Checklist	§5.2
A.3 Facility Validation Checklist	§5.2 and §5.3
A.4 Test Execution Checklist	§5.4
A.5 Monitoring and Sampling Checklist	§5.4
A.6 Analysis and Method Detection Limit Checklist	§5.4.6

*The QMS includes items from 46 CFR §162.050 and ISO 17025.

Checklist data fields are effectively the column headers that are completed for each item in the checklist, but completed at different stages in the audit.

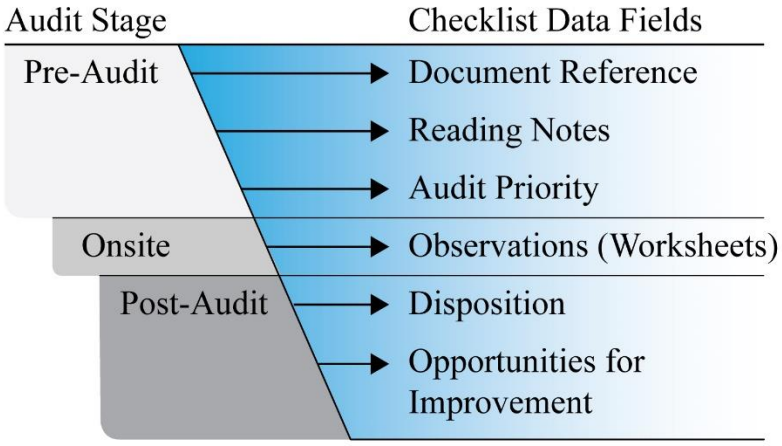

Figure 3. Audit stages and checklist data fields.Following the site visit, auditors will compare the objective evidence compiled in the checklists, and assess conformance to the test facility's procedures and the requirements of TA testing.

[3] Throughout this section, terms relating to audit protocol specifics are in bold and italicized type when they first appear.

Auditors will complete several ***Worksheets*** in support of the checklists:

1. Water Source
2. Analysis of Organisms ≥50 µm
3. Analysis of Organisms ≥10 and <50 µm
4. Analysis of Organisms <10 µm
5. Method Detection Limit

Worksheets provide auditors a template to: describe (potentially) multiple sources of test water, verify the numerous requirements associated with the biological analyses, and calculate the method detection limits for all of parameters measured. The *Water Source* worksheet is completed in support of the *Challenge Water Checklist* and intended to facilitate observations onsite; other worksheets are in support of the Analysis and Method Detection Limit Checklist.

Sections 5.1 through 5.6 provide prescriptive steps for each audit task in Figure 3.

5.1 Pre-audit: Initial Preparation, Document Review, and Agenda

Step 1. The Lead Auditor is tasked with executing IL audits, which may focus on one or more TFs (note, these steps may also apply to other sub-laboratories). Specifically, the Lead Auditor is responsible for assembling the audit team, scheduling and conducting the audit, and completing the audit report.

Step 2. The Lead Auditor will reach out to the IL leadership, indicating interest in an audit and potential dates for an onsite visit. The Lead Auditor will also request current versions of the IL and TF SOPs, Quality Management System (QMS) documents (including the Quality Management Plan, the Quality Assurance Project Plan, and any QMS accreditations), internal validation studies, requests for alternate procedures (i.e., 46 CFR §162.060-10(b)1 requests), and, importantly, the ***Test Quality Assurance Plan*** (TQAP) for the testing that occurs during the site visit.

Step 3. A site visit should be arranged with IL and TF management to coincide with the following operations: challenge water preparation, uptake sampling, and discharge sampling. Ideally, the site visit will also include a day prior to or after test operations for meetings with management, quality personnel, and interviews with selected staff, along with any necessary security and safety briefings.

Step 4. In addition to requests to the IL and TF, the Lead Auditor will also request documentation from MSC, including results of previous audits, previous TA applications, and other relevant communications (such as requests for alternate procedures).

Step 5. The audit team is tasked by the Lead Auditor to review relevant portions of the documentation for objective evidence, so that among all reviewers, *all* the documentation is critically reviewed. The reviewers will address the relevant items in the checklists (Appendix A), noting the location(s) in the documentation (document identification and page or section number) where each item is described. Auditors will make notes if applicable (or write "N/A" for not applicable or not available). Finally, the auditors will subjectively rank the priority—low, medium, or high—for observing and discussing each item during the onsite audit. The audit team will prepare a list of documents received and reviewed; this list will be included in the final report.

Step 6. Upon completion of the document review, the Lead Auditor and audit team meet to discuss to review findings, and based upon the issues noted in the document checklists, set *specific audit goals*.

Specific audit goals, e.g., may include witnessing an analysis procedure, sample processing, or discussions of procedures for labeling and tracking samples. The audit team may also request additional documentation from the TF to address gaps or questions that occurred during the document review.

Step 7. The audit team will develop a draft agenda for the site visit, using the estimated days and times of major test operations. The Lead Auditor will send the agenda to the IL and TF management in advance of the meeting, so that their management has the opportunity to propose changes.

5.2 IL & TF Management Meetings and Staff Interviews

Step 1. While onsite, the Lead Auditor and the audit team will meet with the IL and the TF management. IL management will include the IL Project Manager. TF management will include the TF director, the Quality Officer, and others as required.

Step 2. During the meeting, which ideally occurs at the start of the site visit, the Lead Auditor will convey the general goals of the audit (See Section 3 above), but also the specific audit goals as defined in during the document review.

Step 3. The Audit Team will review the internal studies, validations, and personnel qualifications of the test team. The Lead Auditor will request that the IL and TF management summarize any recent changes to TF equipment, SOPs, and personnel. Recent changes include those made since the IL certification or, if applicable, since the previous audit.

Step 4. The Lead Auditor will also request that the Quality Officer summarize data quality studies, including trending reports (e.g., control tank discharge organisms concentration measurements over time), *Data Quality Indicators* (DQI), and examples of personnel training and qualification tests, such as counting accuracy relative to an experienced microscopist.

Step 5. Auditors will review the validation studies performed by the TF. At a minimum, the ETV Protocol requires the following validation studies:

- ETV §5.2.1: Augmentation of challenge water.
- ETV §5.2.1.3: Homogeneity of the challenge water at the system entry.
- ETV §5.3.1: Similarity between the treatment and control piping and tanks.
- ETV §5.3.2.4: Representative sampling.
- ETV §5.3.3: Injection of organisms and additives.
- ETV §5.4.6.3: Counting accuracy when counting concentrated or large-volume samples.
- ETV §5.4.6.4: Organism mortality and hold time.
- ETV §5.4.6.4: Preservation agent used for fixing samples.
- ETV §5.4.6.4: Initial and on-going measurements of false negatives and positives.

Step 6. During the meetings, the IL and TF management may provide a tour of the test facilities, laboratory spaces, and major testing equipment and analytical tools. While on this tour, the participants should discuss cleaning and decontamination approaches as these are easily overlooked by auditors during actual tests, where focus is directed on sampling and analytical procedures.

5.3 Challenge Water Preparation

Step 1. The auditing team will schedule their visit to witness the preparation of challenge water for a BE test. This may occur during the day of the uptake fill (if a suspension is added directly during uptake), but more frequently, this occurs the day prior to the uptake fill. Auditors will complete the *onsite* auditing checklist for challenge water (Appendix A.1).

Step 2. Auditors will focus on three aspects of the challenge water: 1) water sources and salinity manipulations, 2) addition of abiotic materials, and 3) addition of living organisms. For these items, auditors will observe the timing of the manipulations (e.g., when is the source tank filled? what is the quantity of organic matter added? what is the process for dissolving soluble compounds, and how are solids kept suspended during injection?). Auditors will list information and short descriptions in the ***Water Source Worksheet***. Auditors will only be onsite to witness the testing of one water type, but the following information should be verified in interviews with the TF management and personnel:

- **Water Source:** Define the location of the source water and describe the characteristics of the water source (e.g., hypertrophic lake, tidal estuary, groundwater). List all water sources contributing to the test water, if multiple water sources are used.
- **Volume:** Define the volume and the method of measuring volume (e.g., flow meter totalizers); if multiple water sources are used, describe each volume separately.
- **Date Collected:** Report the actually date of water collected and the time relative to the test start (e.g., "Collected 30 h prior to experiment start and held in the open-air source water tank").
- **Conveyance:** Describe the approach used to transport water, if collected remotely; whether collected remotely or locally, describe the pumping and piping used to transfer water into the source tank or the main ballast line.
- **Salinity Manipulations:** Describe the approaches used to manipulate test water salinity, including volume of brine or freshwater added.
- **Holding Conditions:** If test water is collected or processed prior to testing, describe the holding conditions, such as mixing or agitation frequency and continuous measurements (e.g., temperature, dissolved oxygen, etc.)
- **QA/QC:** Review the TF's approaches to test water used to verify temperature and salinity.

Step 3. Auditors will catalog all organisms added to the test water, including enrichment cultures of natural organism, monocultures of STOs, and concentrated organisms from natural waters. Auditors will document the procedure for enrichment directly in the source tank, if performed. Additionally, auditors will complete a narrative, addressing the following items:

- **Validation Studies:** Request validation studies examining the survivability of organisms added to the test water, and summarize the study methods and findings.
- **Mixing and Injection:** Describe the process for homogenizing the additives and injecting them into the test water. If additives are added to a source water tank, describe how the tank is kept mixed prior to and during testing.

Step 4. Similar to biological components, auditors will list all abiotic components added to the test water, describing their sources, quantities used, and QA/QC procedures for testing the additives. Additionally, auditors will complete a short narrative, addressing the following items:

- **Validation Studies:** Request validation studies examining the impact of the additives chosen on the ambient, biological community. Determine whether the additives used could impact treatment processes. For example, could additives affect oxidant demand, UV transmittance, or filter efficiency in a manner that differs from natural waters.
- **Mixing and Injection:** Describe the process for homogenizing the additives and injecting them into the test water. If additives are added to a source water tank, describe how the tank is kept mixed prior to and during testing.

5.4 Uptake and Discharge Operations

Step 1. The test system should be cleaned prior to (and between) test operations, and, while onsite, auditors will observe cleaning procedures, which are in place to guard against contamination of the infrastructure (ETV §5.3.1). In this narrative, auditors will describe the cleaning approach and validation testing performed to verify that retention of organisms does not influence test results.

Step 2. The auditing team will observe uptake and discharge operations, during which, they will complete the *onsite* Operation and Sampling Checklist (Appendix A.2). Auditors will not interfere or distract test personnel during this time. Auditors should be allowed to photograph equipment and procedures, though all personnel should be aware that the photo documentation will not be publicly distributed (and personnel will not be identified in the images). Auditors will use the images to assist in narrating test operations or used to prompt follow-up discussions with the TF management.

Step 3. Auditors will produce a short narrative, describing the following aspects of the test operations:

- **Project Management:** Describe observations during pre-test meetings, indicating whether the test team reviews the set points, objectives, and required measurements (ETV §7.1). Verify that the Vendor is not directly involved in testing (ETV §2.1).
- **Environmental, Health, and Safety:** Verify that TF management inform onsite personnel and visitors of potential hazards and note relevant observations. Determine whether the onsite staff follow the environmental, health, and safety requirements stated in their documentation (ETV Chapter 2).

Step 4. The audit team will verify the key sampling information in the TQAP, including the sample locations, the volumes sampled, whether samples are discrete or continuous, and if the samples are processed and handled in ways to avoid mortality of aquatic organisms. This includes avoiding high-pressure differentials across the filter bag or plankton net, proper documentation on chain of custody forms, keeping samples containers out of direct sunlight, etc. (ETV §4.3.1.3).

Step 5. For each sample collected, auditors will produce a short narrative, indicating the following aspects of sampling:

- **Sampling Equipment:** For each sample point, describe the sample port, valves, flow control equipment, tubing, plankton nets and their secondary containers, sample vessels, and coolers used for holding or transporting samples. This description should consider whether in-line sampling is performed following requirements (ETV §5.3.2.4 through §5.3.2.7).

- **Sample Source and Processing:** Indicate whether samples are directly collected from the main piping or from a secondary container (e.g., a 1- or 3-m^3 tank used for organisms >50 µm). For each sample, record the sample volume of whole water, and if the water is filter-concentrated, indicate the method used (e.g., plankton nets), and the volume of the concentrated sample. List in the narrative the methods used to measure and verify sample volumes (e.g., container gradations, flow meter totalizers, mass, or other methods).
- **Sample Storage and Transport:** Observe and describe the processes for controlling the samples on site and the process for transferring samples to the analytical personnel (e.g., chain-of-custody procedures, ETV §7.2). Note the sample start and end times, the time of storage, and the time of arrival at the analytical laboratory.

Step 6. Auditors will document the procedures for processing treated water prior to discharge, indicating whether both treatment and control water are processed and the procedure for processing effluent and handling other waste generated during testing (ETV Chapter 9).

5.5 Sample Processing and Analysis

Step 1. The auditing team will observe the analysis of samples collected during the uptake and discharge operations. Auditors will complete the Analysis and Method Detection Limit checklist (Appendix A.5).

Step 2. Auditors will calculate the MDL of all biological parameters using the Method Detection Limit Worksheet. The MDL considers that the minimum unit of detection is one organism.

5.6 Audit Wrap-Up and Report Generation

Step 1. All checklists should be completed prior to leaving the site, and auditors should quickly finalize notes and observations, checking their narrative statements for clarity and accuracy. All information should be transcribed into digital form, such as in a spreadsheet or database.

Step 2. Auditors will also catalog their photo/video documentation, such that the digital records are referenced to the appropriate place in the checklist. The originator of the image file should briefly describe the image, e.g., indicate the location of the photo/video, the relevant procedure or the equipment description, and the purpose and relevance of the image.

Step 3. Following the onsite audit, the Lead Auditor (or delegate) will merge comments from all auditors, review the checklists, worksheets, and narrative comments. Where auditors were not in consensus, the Lead Auditor will review the items, consult the auditors, and resolve differences. If differences cannot be resolved, the Lead Auditor will note the team did not reach consensus. In this situation, the Lead Auditor may note the disagreement in the overall findings.

Step 4. The final report will include:

- The list of documents reviewed.
- The consensus checklist.
- A summary of the overall findings, including a list of non-conformances to the requirements and observed deviations from the TQAP (note that these should be documented in any TA application that cites the observed testing).
- The opportunities for improvement.

Step 5. The final report should be reviewed by all auditors prior to submission. Following submission of the final product, the report text, photo/video documentation, digital scans of field notes, and other relevant records should be archived for at least five years.

Step 6. Under ISO/IEC 17025, audit reports are part of the quality management process, and the test facility (and likely the IL as well) will want to receive a copy of the audit report in order to act on any deficiencies, non-conformances, and opportunities for improvement. Based on the experiences of the audit teams during this project, the TF will also request a preliminary debrief at the conclusion of the onsite visit. The audit team should be prepared to respond to these requests.

6 RECOMMENDED ADDENDA TO ETV PROTOCOL

Following the four TF site visits, review of TF documentation, and discussions with TF personnel, the project identified several issues that could be clarified in either addenda to the ETV Protocol or added to subsequent test requirements. The issues are briefly described in the sections below.

6.1 Guidance on Additive Selection

Additives used to supplement dissolved and particulate matter are known to have *direct* impacts on treatment technologies, but they also have *indirect* impacts on the biological community. Importantly, additives will increase the concentration and activity of heterotrophic bacteria (First et al. 2014), and increased bacterial activity will affect oxygen demand, but also nutrient cycling and sequestration (Gardner et al. 1994). Turbidity increases with bacterial concentration, and abundant bacteria may drive changes in concentrations of bacterivorous protists, which can respond rapidly to shifts in prey abundance (Kim et al. 2014). A concern is that changes occurring—directly or indirectly—in response to additives affect the susceptibility of organisms to treatment. The TF could (and should) track changes between the community structure in the uptake water and control tank discharge. The ETV Protocol requires additives are validated, and that the "TF must assess the effect of additives on the ambient and test organisms" (§5.2.1.2). **The project recommends that required validations of additives are expanded to include measurements of indirect effects, such as dissolved oxygen, and pH, in addition to tracking changes in the concentrations of major taxa.** To accomplish this, TFs should conduct validation experiments comparing test water with and without additives. Test water should be prepared following procedures used in TA testing: If ambient organisms are concentrated, transported from remote locations, or supplemented with STOs, these same approaches are used in validation tests. The taxonomic diversity and the concentration of organisms must meet those specified in the ETV Protocol. The validation should use volumes and hold times similar to TA testing, however, mesocosm-scale (1-5 m^3) may be sufficient. Following an incubation period, test personnel measure water characteristics and organism assemblages in the amended and unamended treatments. Finally, test personnel should treat sample aliquots with a disinfection technology (e.g., UV radiation, chlorination, etc.), similar to those used by BWMS. Such experimental manipulations may be performed at the bench-top scale. This experimental validation tests the null hypothesis that additives do not affect the survivability of organisms. This expanded validation be performed for each test condition (hold time, additives used, water type and source, etc.) and periodically, such as every test season.

6.2 Standardized Procedures for Validation Studies

Following guidance in ISO/IEC 17025, non-standard methods should be validated. While the ETV Protocol provides general requirements, the materials, most reagents, sample handling and processing techniques,

and equipment that are not specified in the ETV Protocol will be unique to the TF. Validation studies must be designed to yield statistical power to verify the method is appropriate in that it meets the defined requirements for, among others:

- Analytical uncertainty (false positive and negative error rates).
- Mortality or loss rate.
- Concentration efficiency.
- Equivalence of control and treatment tracks.

The ILs could define test regimes, choose treatments, and then set and justify pass/fail criteria. However, guidance from the USCG would be authoritative. For these reasons, **the project recommends the USCG provide examples of validation studies and list guidelines for acceptance.** USCG should consider participating in the Global TestNet forum to observe the issues and concerns raised by the TFs, as it is an appropriate venue to relay new information or clarify requirements.

6.3 Data Quality Indicators for Microscope Counting

Assays for the key measurements in BE testing require manual microscopy. As such, analyst training, skill, *and stamina* are central considerations. While a reference standard is not available (as discussed above in Section 2.1), the quality of microscope-based analyses may be demonstrated by one of the following approaches.

6.3.1 Automated Tools for Benchmark Measurements

Rapid analytical tools designed for compliance testing are now readily available. For example, optical-based instruments quantify—by direct counting—organisms in both the ≥50 µm and the ≥10 and <50 µm size classes. These instruments are marketed as compliance monitoring devices for rapid, shipboard analyses (MEPC 74/INF.18). While these optical-based approaches are simplifications of manual microscopy, they may provide a baseline value. With a record of side-by-side measurements, the factor of agreement could be determined, so that for ongoing analyses, measurements outside of the factor of agreement could be flagged, investigated, and potentially samples could be reanalyzed. **The project recommends that TFs investigate automated instruments that could potentially provide a benchmark for manual counts.**

6.3.2 Quantifying Measurement Uncertainty

Multiple samples are analyzed in short time periods, and most TFs dedicated multiple analysts to work on samples concurrently. Because of this, TFs should measure variation among analysts and compare it to inter-replicate variation (ETV §5.4.6.5). To quantify inter-analyst variation, a single sample chamber is counted by multiple analysts, who are blinded to each other's readings. Inter-replicate variation is the difference among multiple analyses of subsample drawn from a homogenized sample. Both inter-replicate and inter-analyst variation should be measured in validation studies (e.g., First et al., submitted), but inter-replicate variation may be tracked over time from historical records of sample analyses TFs should produce an error budget for all analyses of organism concentrations. The error budget accounts for all the sources of uncertainty and estimates their magnitude by empirical studies. While some TFs had established criteria for training, none provided a study of measurement uncertainty that encompassed all aspects of the BE measurement process. Ideally, each TF should document the minimum detection limit and measurement uncertainty associated with each independent trial. These empirical estimates of uncertainty should be used to specify data quality indicators (DQI) and objectives, e.g., to flag instances where variation among replicates exceeds objectives or the historical measurements of variation.

6.3.3 Video Recording of Counting For Verification

Video microscopy is commonly available for modern microscopes, and it can be used to create a record of counting for both organisms ≥50 µm and organisms ≥10 and <50 µm. While it is not feasible in multiple analyses to review all counting video, it certainly would be a useful tool for periodic data checks, for training, and for harmonization among multiple analysts. **The project recommends periodic review (annually or more frequent) of video recordings of microscope analyses.**

6.4 Simplified Approach for Enumerating Toxigenic *V. cholerae*

All TFs used approaches for toxigenic *V. cholerae* that differed from the approach defined in the ETV Protocol. The required approach is one several described by Huq et al. (2006). Of the approaches, it is likely the most difficult to perform. First, the ETV Protocol does not specify whether a filtration step is required or if a enrichment step is needed, but Huq et al. (2006) recommend an initial concentration step, where the concentrated particulates are added to APW media. A secondary culturing is performed with thiosulfate citrate bile salts (TCBS).

The ETV Protocol requires that cultures on TCBS are "…purified, and inoculated with 2.5% yeast extract and nalidixic acid and fixed after incubation overnight." (Section 5.4.6.8). The purpose of this step, is to distinguish between actively growing and non-growing individuals. In a rich media, active cells would grow then divide, but nalidixic acid prevents cell division, so active cells become enlarged. Colonies appearing on TCBS agar likely originated from a single individual (a "colony-forming unit"), so the portion of actively growing individuals depends more on the growth phase and location within the colony (e.g., Dietrich et al. 2013) than its viability in ballast water.

The fluorescently labeled antibodies for O1 and O139, rather than DNA oligonucleotides, are used for identification, so the approach is best described as a direct-fluorescent antibody-direct viable count (rather than a DNA colony dot blot hybridization). The assay, as described in Huq et al. 2006, requires numerous steps that are time- and temperature-sensitive, a suite of reagents, including fluorophore-antibody conjugates.

The project recommends using methods described by Huq et al. (2006). First, 100 mL of sample water should be concentrated on a sterile filter, which is incubated overnight in APW. Subsample from the pellicle of the liquid culture is spread on TCBS plates. Suspect colonies are identified based upon morphology, and processed for downstream analyses as described in Huq et al. (2006), including gene amplification or biochemical, metabolic assays.

7 CONCLUSIONS

The land-based test procedure described in the ETV now approaches its tenth year, and ILs and their TFs have implemented and executed its procedures such that 22 BWMS have received Type Approval (Marine Safety Center, www.dco.uscg.mil, accessed: 24-Oct-2019). Going forward, both TFs and the USCG could make improvements to strengthen the TA processing. Periodic technical audits are a key element for forthcoming testing, and the audit protocol described herein would assure the test methods are followed, such that a BWMS—regardless of the TF conducting the testing—receive a rigorous evaluation, demonstrating its suitability to all stakeholders.

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APPENDIX A. AUDITING CHECKLISTS

*These checklists are intended to aid the audit process of Test Facilities (TF) performing land-based, biological efficacy testing for Independent Laboratories (IL) approved by the U.S. Coast Guard. These **six checklists** are used to record objective evidence throughout the audit process, from the pre-audit document review to the final disposition. Certain checklist items refer to **worksheets**, which are used to create an inventory of sources waters, to verify numerous requirements of analytical techniques, and to calculate method detection limits. A reference table from the ETV Protocol (EPA 2010) is also included.*

Contents:
The following checklists each provide multiple items for consideration.

1. **Quality Management System (QMS)**
2. **Challenge Water Checklist**
   Includes:
   Worksheet: Water Source
3. **Facility Validation Checklist**
4. **Test Execution Checklist**
5. **Monitoring and Sampling Checklist**
   Reference: Parameters Table (ETV Table 11)
6. **Analysis and Method Detection Limit Checklist**
   Includes:
   Worksheet: Analysis of Organisms ≥50 µm
   Worksheet: Analysis of Organisms ≥10 and <50 µm
   Worksheet: Analysis of Organisms <10 µm
   Worksheet: Method Detection Limit

Instructions:
Pre-Audit Document Review
Each checklist contains a list of items to assess for conformity, and each of these items should be addressed by the test facility documentation. During the pre-audit document review, address each item in each checklist for:

- **Documentation Reference**
  Identify the document(s) and location(s) within where the item is described. If not addressed, indicate Not Available with "N/A".
- **Notes**
  Note any questions, ambiguities, or imprecise language in the description of each item.
- **Audit Priority**
  Rank the priority of the item—either Low, Medium, High—for onsite observations and discussion with TF/IL personnel. Following the pre-audit document review, create a separate list of the high priority criteria to observe onsite, as it is difficult to page through the complete checklist in the field.

One or two criteria per page allows room for notes, sketches, and reference to any additional documentation (reports supplied onsite, video, photographs, etc.).

Onsite Audit

While onsite, the procedures described in the test facility documentation should be observed. Those items cited as **high priority** during the pre-audit document review should be a focus of observations to resolve any discrepancies or questions as to how the item conforms to the requirements.

- Discuss the checklist items with key management and technical individuals.
- Investigate items ranked as high priority to document approach in question, follow up on other queries as possible.
- Complete onsite worksheets, briefly document observations and discussions with personnel, and refer to photo/video documentation, as applicable.

Post-Audit Review

Following the audit, convene with the audit team to collate and discuss observations. Summarize written narratives and observations, and (if necessary) note where auditors *were not* in consensus.

- **Disposition**
  Address each checklist item for conformance to the requirement. Even if unable to directly obtain objective evidence or assess some items, provide a general impression of conformance or state that the item could not be assessed so as to flag the criteria for future audits.

- **Opportunities for Improvement**
  Identify areas which could result in improvements, especially with regards to efficiency of test operations, analytical processes, or data quality.

For all items, indicate whether an approved alternative method or a validation study is available and use "N/A" to indicate "not applicable" or "not available".

A.1 Quality Management System

1 **Management Requirements of ISO 17025**

- The test facility is accredited to ISO 17025 or other Quality Management System (not required, but if so, obtain scope)
- A member of the staff is appointed as quality manager (however named), who has the responsibility and authority for ensuring that the management system is implemented and followed at all times; he or she has access to the highest level of management at which decisions are made on facility policies or resources (ISO 17025 §4.1)
- The facility employs a process for continuous improvement through the use of quality objectives, audits, data analysis, preventive and corrective actions (ISO 17025 §4.10)

2 **Test Facility Personnel**

- A list of key personnel, which provides affiliations (e.g., with a nearby university) and associated responsibilities, is included for individuals with the following responsibilities: test director, site operations manager, sample collection and handling personnel, quality assurance/quality control (QA/QC) officer, and at least one analyst for each of the three biological size classes; all personnel must be employed by or under contract to the independent laboratory (IL) or test facility (TF)
- Test operations staffing is consistent with the requirements specified in the QAPP; the minimum expectation for a facility incorporating automated data logging and control is to employ: 1 onsite test director, 2 operations engineers, 1 QA/QC officer, 1 sample handling staff member, 2 analysts for organisms ≥50 µm, 2 analysts for organisms ≥10 and <50 µm, and 1 analyst for organisms <10 µm

3 **Technical Requirements of ISO 17025**

- The facility examines and considers the factors that affect measurement uncertainty (e.g., equipment, training, environmental conditions) and uses this information in the development of methods, training of personnel, and selection of equipment used in their test and calibration protocols (ISO 5.1)
- Environmental conditions are monitored, controlled, and recorded as required by relevant specifications and protocols to ensure measurement quality. These conditions may include, for example, biological sterility, dust levels, temperature, electrical supply, or other parameter as appropriate to the quality of the measurement. Tests and calibrations are stopped when environmental conditions may affect the results (ISO 5.3)
- The facility maintains up-to-date instructions, standards, manuals, and reference data relevant to the work of the facility, which are accessible and available to all technical personnel (ISO 5.4)
- The facility maintains a record of any deviations from testing parameters and calibrations, and it includes technical justifications for the deviations, facility authorization, and customer approval (ISO 5.4)
- The facility selects appropriate methods that have been published as a standard or specified by a manufacturer. Where necessary, the facility may develop or adopt other non-standard methods deemed appropriate for use in testing or calibration, but they must be validated. Such validation must address the uncertainty of the method or the procedure for estimating uncertainty, and it must include a statement regarding the method's suitability for the intended use (ISO 5.4)
- The facility incorporates methods to check calculations and accuracy of data transfer when processing test and calibration data (ISO 5.4)

- Records are maintained for each item of equipment and its software significant to the tests and/or calibrations performed; records shall include unique identifiers (e.g., model and serial number), calibration dates, and maintenance history (ISO 5.5)
- The sampling procedures include recording of test parameters that require control to ensure the validity of the test (e.g., flow over time, volumes, or sample concentration factors) (ISO 5.7)
- The facility has quality control procedures for monitoring the validity of tests and calibrations undertaken; the resulting data are recorded and reviewed in such a way that trends are assessed, and where practicable, statistical techniques are applied to monitor and quantify confidence in the results (ISO 5.9)
- The facility documents any deviations to the test processes and includes a description of all deviations and their impact on results in the test report (ISO 5.10)

4 **Quality Assurance Project Plan (QAPP) Requirement**

- The test facility has a Quality Assurance Project Plan (QAPP) for ballast water management system (BWMS) testing that is regularly reviewed and updated (as defined by the facility's Quality Management Plan [QMP])
- The QAPP identifies and defines all relevant procedures, measurement methods, and test protocols (i.e., the QAPP indicates all standard operating procedures [SOPs] are provided, likely as an appendix or in other external references)
- The QAPP describes the methods and statistical analyses used to assess data quality (including criteria for data quality indicators [DQIs]) and acceptance limits for the following indicators: representativeness accuracy, precision, bias, comparability, and completeness
- The QAPP indicates the use of spikes, blanks, and detection limit indicators as quality checks where applicable
- The QAPP identifies the processes for data entry, data analysis, data management, and data security
- Overall, the QAPP provides a thorough description of the experimental approach employed by the facility that includes the objectives and purpose of the testing, the apparatus and processes used to perform the testing (from installation through decommissioning), and the methods that establish data quality and accuracy of the reported results.
- The QAPP identifies all technical system audits (TSAs) and performance evaluations (PEs) to be performed, who will perform these audits, and whom will receive the audit reports
- An example Test Plan or Test Quality Assurance Plan (TQAP) is provided

A.2 Challenge Water Checklist

1 **Source water origin**
Source waters must be from natural fresh, brackish, or sea waters **(complete *Worksheet: Water Source*).**

2 **Salinity modification**
If the salinity is artificially modified, the facility must provide data showing the concentrations, diversity, and condition of organisms will not be affected by the modification.

3 **Biological Additives**
The majority of challenge water organisms are ambient organisms, not standard test organisms (STOs). Data are provided to show the percentage of ambient organisms in challenge water, the percentage of added or injected organisms that are harvested from ambient waters (e.g., with a plankton net), and the percentage of added or injected organisms that are STOs.
(Narrative: Describe validations studies)

4 **Introducing Biological Additives into the Test Water**

- Any method used to add or inject organisms must minimize, to the extent possible, organism mortality as a result of its addition or injection mechanism; if organisms are added to the test water, the TF references data—either internal TF or published data—showing that the method results in a predictable and suitable density of robust organisms in the challenge water.
  (Narrative: Describe mixing and injection studies)
- The test facility (TF) injects organisms in a time-averaged manner (i.e., constant flow injection over the entire test); the introduction method results in a well-mixed and uniform distribution, spatially and temporally, of organisms within the challenge water and at its introduction at the point of treatment or tank intake.
- The TF provides documentation to show that the location of organism injection is appropriate (e.g., it is 10 pipe diameters upstream of the sampling location, so that samples collected are well mixed).

5 **Non-Biological Additives**

- The TF should provide a description of the augmentation process that indicates the method, location, and equipment used for delivery of water quality additions.
  (Narrative: Describe mixing and injection studies)
- Where augmentation of water chemistry occurs, the selected materials should minimize, to the extent possible, biocidal or growth responses by the ambient organisms.
- The TF must provide data from validation studies showing the materials used to augment water do not increase mortality (either ambient organisms or STOs).

6 **Introducing Non-Biological Additives into the Test Water**

- Any injection of any additives in TF piping system should be located and implemented such that it allows for sufficient mixing after injection.
- The augmentation of water quality constituents occurs in a time-averaged manner (i.e., constant flow injection over the entire test, or pre-mix of challenge waters should maintain a uniform suspension over the course of the test cycle).
- The facility produces experimental validation or computational fluid dynamic models that the injection system results in challenge water constituents that are well-mixed. If no computational fluid dynamics modeling results are used to demonstrate appropriate mixing, the location(s) should be at least 10 pipe diameters upstream of any sampling apparatus.
  (Narrative: Describe mixing and injection studies)

Worksheet: Water Source

Source waters must be from natural fresh, brackish, or sea waters (unless alternatives are approved). Assess each water source for conformance to TQAP. The provenance of the source water(s) is documented and includes date and time of collection, source location, transport history (if not locally sourced), storage history, salinity, and any other data required by facility SOPs, QAPP, and TQAP.

For each of the water types used in testing, indicate or briefly describe the following:

- Source (provenance)
- Volume
- Date Collected
- Conveyance
- Quality testing
- Salinity manipulations (brine, freshwater additions)
- Storage history
- Mixing or homogenization approach
- Other manipulations

A.3 Facility Validation Checklist

1 **Test Facility Validation**

- A validation study is conducted that demonstrates any differences in biological efficacy and water chemistry between the control and treatment tank discharges are minor. The study occurs when a BWMS is not used, so results should validate that both control and "treated" water are equivalent in:

  - Water characteristics (temperature, salinity, dissolved oxygen, total suspended solids, etc.),
  - Water flow rates and pressures, and
  - Living organism concentrations.

- The project management, onsite vendor representatives, and test personnel meet to review personnel responsibilities and roles. Vendor representatives do not interfere during testing. All onsite personnel are briefed on the environmental, health, and safety concerns. Those onsite comply with safety requirements. **(Narrative: Describe project management and health and safety compliance.)**

2 **Cleaning Validation**

- Procedures are described to validate the effectiveness of cleaning lines (e.g., using an inspection technique to examine the residual level of a biocide or to count organisms). **(Narrative: Describe cleaning approach and validation studies)**

- If no cleaning occurs between ballast operations, the operational sequence is designed to prevent cross contamination. Such a sequence could be: in uptake operations, water flows through control piping prior to treatment piping, and in discharge operations, treated water flows through the piping prior to control water. A small amount of the control discharge volume can be used to flush common piping if it is discarded from the measured control volume.

3 **Verification of Operational Parameters**

- Test parameters met the requirements defined in the TQAP for:
  Manipulations of source water,
  Water flow rates and tank volume,
  Sample flow rates and sample volumes,
  Sample collection locations, and
  Sampling procedures.

- Sensor maintenance and calibration is performed as described in the TQAP.

- All deviations to the TQAP (including deviations from the QAPP and SOPs) were documented and reviewed to assess impacts on the validity of the test cycle.

- The BWMS control and monitoring functions operated correctly; any alarms were noted, and ballasting operations were completed within TQAP requirements for a valid test.
- Any deviations to the procedures were documented and their effect on the experimental result assessed.

A.4 Test Execution Checklist

1 **Test Water Characteristics (Biological)**

- Organism concentrations are:
  100,000 m^{-3} for organisms ≥50 µm,
  1000 mL^{-1} for organisms ≥10 and <50 µm, and
  1000 mL^{-1} for bacteria (as culturable, aerobic, heterotrophic bacteria).
- For organisms ≥50 µm as well as ≥10 and <50 µm, assure there are at least 5 species across 3 phyla.

2 **Test Water Characteristics (Non-Biological)**

- Salinities are:
  <1 PSU for fresh,
  10-20 PSU for brackish, and
  28-36 PSU for marine tests.
- Concentrations in challenge water are at least:
  6 mg L^{-1} as DOC for DOM,
  4 mg L^{-1} as POC for POM,
  20 mg L^{-1} for MM, and
  24 mg L^{-1} for TSS.
- Test water temperature ranges from 4-35°C.

3 **Test Water Flow and Storage**

- The documentation package provides a map of the site layout, piping and instrumentation diagrams, identification of tanks, pumps, sensors, valves, etc. Facility documentation describes the process to start and stop the ballast flow as well as to control the flow rate to a specified set point.
- The movement of water between various facility subsystems is described. Facility documentation includes a complete list of main flow control valves, including the type of valve and the type of actuation (manual vs. automatic) used to control the valves. The facility demonstrates an ability to control ballast operations with a facility control methodology that logs operations such as the start time of the main ballast pump, the position of valves, etc.
- The instantaneous flow rates and pressures are measured and data are logged using calibrated flow and pressure sensors. Calibration logs are maintained for instrumentation and gauges used in monitoring water movement and storage.
- The method for ballast uptake and discharge operations (such as simultaneous vs. sequential uptake and discharge) are defined and at least 400 m^3 are available for each test cycle (split between control and treatment tracks).
- Both treatment and control tank volumes are each ≥200 m^3, and both tanks can hold treated water for a minimum duration of 24 h.

A.5 Monitoring and Sampling Checklist

1 **Water quality monitoring**

- The parameters listed in the Parameter Table (from ETV Tables 9 and 11, see below) are sampled and measured in control and treatment waters upon both uptake and discharge.
- All analyses above shall include 2 procedural blanks (except none required for dissolved oxygen) and 3 sample replicates for each test cycle.
- Analyses are performed within the time frame required by the SOP; the IL should approve if samples are stored or shipped for outside analysis.

2 **Biological sample volumes**

The sample volumes collected for biological samples are consistent with the method requirements, and the volumes measured are consistent with the TF's procedures (SOPs, QAPP, and TQAP).

3 **Sample handling, transport, and hold time**

Samples are stored and transported under appropriate conditions for subsequent analysis, and analyses are performed within the appropriate time frame, as identified in the relevant facility SOPs and QAPP.
(Narrative: Describe sampling equipment, sample processing, storage and transport.)

4 **Sampling Apparatus Design and Implementation**

- The sample port diameter for sampling organisms ≥50 µm has been sized 1.0 to 2.0X the isokinetic diameter (D_{ISO}) as calculated by the following equation, which is discussed in the Quality Assurance Project Plan (QAPP). D_{MAIN} is the internal pipe diameter of main ballast pipe; Q_{ISO} is the sample flow rate; and Q_{MAIN} is the main ballast flow rate. Deviations from this size are justified with modeling or empirical data.

$$D_{Iso} = D_{Main}\sqrt{\frac{Q_{Iso}}{Q_{Main}}}$$

- The sample probe entrance axis is aligned to the main ballast flow and has chamfered edges. **Note:** the probe should be oriented such that its entrance leg flow is parallel to the main pipe flow and concentric to the larger pipe; the length of the straight section of the sample probe can vary, but it should not be less than one pipe diameter of the sample probe.
- If flow control is used to control the sample flow rate, a diaphragm valve or similar valve type is used when located prior to sample collection device (e.g., net or filter). **Note:** the ETV Protocol has specific guidance to not use ball, gate, or butterfly valves unless they are left in the 100% open position during sampling because they may induce organism mortality.
- The sample probe is placed in the main pipeline at a location where flow is fully mixed and fully developed. For guidance, the sampling point should be installed in the straight part of the system; computational fluid dynamics modeling is recommended to optimize location; at a minimum, the sample port should be located at least 10 pipe diameters downstream of any elbows, valves, or pumps.
- The biological samples are collected on a time-integrated basis over the entire period of uptake or discharge; if the flow rate is not constant, the sample flow rate should be appropriately controlled to be proportional to main ballast flow.

Parameters Table (ETV Tables 9 and 11)

Parameter	Sample volume (minimum)	MDL	Data Quality Objective
Temperature	*In situ via electronic temperature probe*	Not specified	Not specified
Salinity	*In situ via electronic conductivity probe*	Not specified	Not specified
Particulate organic carbon	500 mL	5.5 µM	Procedural Blank: ≤15% PD Sample Replicates: ≤15% RPD
Dissolved organic carbon	25 mL in glass container	20 µM	Procedural Blank: ≤15% PD Sample Replicates: ≤15% RPD
Total suspended solids	100 mL in HDPE or glass bottle	0.1 mg/L	Procedural Blank: <5 times MDL Sample Replicates: ≤10% RPD
Dissolved oxygen	300 mL in glass BOD bottle	Not specified	Procedural Blank: N/A Sample Replicates: ≤5% CV
pH	*In situ via electronic pH probe*	Not specified	Not specified

MDL = Method detection limit
PD = Percent difference [(true concentration - measured concentration)/(true concentration)] x 100%
RPD = Relative percent difference {[absolute value (replicate 1 – replicate 2)] / [(replicate 1+ replicate 2)] / 2} x 100%

A.6 Analysis and Method Detection Limits Checklist

1 **Analysis of organisms ≥50 µm**
- The maximum sample holding time and conditions has been validated and data provided.
- The requirements listed in ETV **§5.4.6.4** are met.
  (Complete "Analysis of Organisms ≥50 µm" Worksheet)

2 **Analysis of organisms ≥10 and <50 µm**
- The maximum sample holding time and conditions has been validated and data provided.
- The requirements listed in ETV **§5.4.6.5** are met.
  (Complete "Analysis of Organisms ≥10 and <50 µm" Worksheet)

3 **Analysis of organisms <10 µm**
- The media used to culture heterotrophic bacteria has been validated for local organisms.
- The maximum sample holding time and conditions has been validated and data provided.
- The requirements listed in ETV **§5.4.6.7** are met.
  (Complete "Analysis of Organisms <10 µm" Worksheet)

4 **Analysis Challenges**
The procedure for detecting and quantifying resting stages, cysts, and other challenges that may be encountered during analysis is described in the TF's documentation.

5 **Method Detection Limits (MDL)**
- The MDL for all analysis, including water quality parameters and biological assays are appropriate. The MDL should be less than the minimum regulated criteria).
  (Complete "Method Detection Limit" Worksheet)

  For the biological assays of the ≥10 µm and < 50 µm and the ≥50 µm size classes, calculate the MDL based on the parameters listed below. This should be assessed for any measurements with different sample volumes (i.e., uptake, treated discharge, control discharge). - Whole water sample volume
  - Concentrated sample volume
  - Analysis volume
  - Sample dilution (if performed)

  MDL is calculated using the following equation:

$$MDL = \frac{1\ individual \cdot Concentrate\ Volume\ \cdot Dilution\ Factor}{Sample\ Volume\ \cdot Volume\ Analyzed}$$

  Below is a theoretical example for ≥50 µm organism in challenge water:
  Whole water sample volume = 30 L
  Concentrate sample volume = 1 L
  Analysis volume = 0.5 L
  Sample dilution (not performed, dilution factor = 1x)

$$\boldsymbol{MDL = \frac{1\ individual \cdot 0.3\ L\ \cdot 1x}{20\ L\ \cdot 0.2\ L} = 0.075\ L^{-1} = 75\ m^{-3}}$$

Worksheet: Analysis of Organisms ≥50 µm (ETV §5.4.6.4)	
1	Time-averaged, in-line samples are used for analysis.
2	Challenge water and control water procedure differs from treated discharge water, in that a larger sample volume is analyzed for treated discharge.
3	Concentration is performed using a 35-µm plankton net. Note that plankton nets should be monitored to ensure no clogging occurs, this may more frequently with smaller pore sizes.
4	Concentrated organisms are rinsed into from the cod-end into a flask, typically 1–4 L volume.
5	Organism-free water with the same temperature and salinity is added to maintain dissolved oxygen concentration in samples.
6	Any additional concentration of organisms is performed using a 35-µm mesh.
7	Analyze samples immediately to minimize organism die-off within the appropriate time (as determined by a validation study); detectable mortality should not exceed 5%.
8	Collect subsamples using a pipette in a manner appropriate to capture swimming zooplankton.
9	Count subsamples in multi-well plates, Bogorov chambers, SR Chambers, or counting wheels.
10	Allow for addition of a narcotizing agent, narcotizing agents are validated for use
11	Use a shallow counting chamber volume so the entire depth is in focus.
12	Use stereo microscope or a compound microscope with 10–40x magnification.
13	Use Lugol's iodine for fixation or other appropriate fixatives as determined from validation studies.
14	Count dead (unmoving) organisms first, touching unmoving organisms with a probe and waiting for 10 seconds for movement. Use this procedure for high concentrations of living organisms (in the challenge water and control discharge.
15	For treated samples, living organisms may be counted directly (rather than counting dead organisms, fixing the sample, then counting both living and dead organisms).
16	Samples from uptake are used to for taxonomy to assure phylogeny requirements are met.
Quality Control	
17	The facility has conducted an onsite validation using heat-killed organisms following the recommended guidance (ETV, footnotes 4 and 5).
18	Initial and ongoing validations are used to monitor Type I (false positive) and Type II (false negative) error.
19	The TF has procedures to mitigate operator-specific biases and fatigue during extended observation periods.

Worksheet: Analysis of Organisms ≥10 and <50 µm (ETV §5.4.6.5)	
1	Concentrate organisms by gently passing sample through a 10-µm mesh and use care not to kill organisms in the process.
2	Use the following fluorophores for a 1-mL sample: 5 µM fluorescein diacetate 2.5 µM chlorofluorescein diacetate (*or others as approved by USCG via 10(b)(1)*)
3	Incubate in the dark for 10 minutes then load into a Sedgewick Rafter counting chamber
4	Examine the sample using the appropriate set of light filters: 465-495 nm excitation 505 nm dichroic mirror 515-555 nm emission
5	Complete sample examination within 20 minutes (considering the 10-minute incubation, total exposure time should be <30 minutes).
6	Organisms fluorescing, moving, or both are scored as living.
7	Photomicrographs are collected in fluorescence and brightfield illumination.
Quality Control	
8	The facility has conducted an onsite validation using heat-killed organisms following the recommended guidance (ETV, footnotes 4 and 5).
9	Initial and ongoing validations are used to monitor Type I (false positive) and Type II (false negative) error.
10	The TF has procedures to mitigate operator-specific biases and fatigue during extended observation periods.

Worksheet: Analysis of Organisms <10 µm (ETV §5.4.6.7)	
1	Use an unfiltered, whole water sample, and stop residual treatment with a neutralizer.
2	Use an appropriate diluent to dilute whole water samples. (e.g., the diluent should be isotonic to the sample water)
3	Positive and negative controls are consistent with the SOPs.
4	Spread 100 µL of the sample (whether whole water or diluted sample) on a plate.
5	Use triplicate plates for each dilution.
6	Incubate plates at 25°C and monitor for colony growth up to 5 days.
7	Count CFU and report the concentration per 100 mL.
8	Perform USEPA Method 1603 or IDEXX Colilert kit.
9	Perform USEPA Method 1106.1 or IDEXX Enterolert kit.
10	Perform a DNA colony blot hybridization to detect ctxA gene.
11	Grow colonies on TCBS, select colonies and incubate in 2.5% yeast extract with nalidixic acid overnight.
12	Use a direct-fluorescence antibody kit (monoclonal antibodies tagged with FITC) and enumerate organisms via epifluorescence microscopy.
Quality Control	
14	Laboratory uses standard microbiological techniques (e.g., sterile techniques) and has standard equipment (e.g., biosafety cabinet, autoclave, etc.).

APPENDIX B. ELECTRONIC CHECKLISTS

Electronic Checklists (Excel)

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