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INTERNATIONAL CONVENTION FOR THE CONTROL AND MANAGEMENT OF SHIPS' BALLAST WATER AND SEDIMENTS, 2004

Guidance on ballast water sampling and analysis for trial use in accordance with the BWM Convention and Guidelines (G2)

1 The Marine Environment Protection Committee, at its fifty-eighth session (October 2008), following the adoption of the *Guidelines for ballast water sampling (G2)* (resolution MEPC.173(58)), instructed the Sub-Committee on Bulk Liquids and Gases (BLG) to develop, as a matter of high priority, a circular to provide sampling and analysis guidance.

2 MEPC 65 (13 to 17 May 2013) approved BWM.2/Circ.42 on *Guidance on ballast water sampling and analysis for trial use in accordance with the BWM Convention and Guidelines (G2)*, as agreed by BLG 17 (4 to 8 February 2013).

3 MEPC 66 (31 March to 4 April 2014) had invited Member Governments and international organizations to submit further information and proposals related to ballast water sampling, analysis and contingency measures to the Sub-Committee on Pollution Prevention and Response (PPR), with a view to further developing and improving the relevant guidance documents and guidelines.

4 MEPC 68 (11 to 15 May 2015) approved the revised *Guidance on ballast water sampling and analysis for trial use in accordance with the BWM Convention and Guidelines (G2),* as agreed by PPR 2 (19 to 23 January 2015), set out in the annex.

5 Member Governments are invited to bring the annexed Guidance to the attention of all parties concerned.

6 This circular supersedes BWM.2/Circ.42.

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ANNEX 1

GUIDANCE ON BALLAST WATER SAMPLING AND ANALYSIS FOR TRIAL USE IN ACCORDANCE WITH THE BWM CONVENTION AND GUIDELINES (G2)

1 INTRODUCTION

1.1 The purpose of this guidance is to provide general recommendations on methodologies and approaches to sampling and analysis to test for compliance with the standards described in regulations D-1 and D-2 of the International Convention for the Control and Management of Ships' Ballast Water and Sediments, 2004 (BWM Convention). This guidance is an updated version of the guidance contained in document BLG 16/WP.4, taking into account advances in research since the document was first drafted, and should be read in conjunction with the BWM Convention, the *Guidelines for port State control under the BWM Convention* (resolution MEPC.259(67)) and the *Guidelines for ballast water sampling (G2)* (resolution MEPC.173(58)). Furthermore, and as instructed by MEPC 64, the sampling and analysis procedures to be used for enforcement of the BWM Convention should result in no more stringent requirements than what is required for Type Approval of ballast water management systems (BWMS).

1.2 This guidance consists of two parts,

.1 a discussion of the principles of sampling, accompanied by a list of recommended methods and approaches for analysis and sampling protocols available for compliance testing to the D-1 and D-2 standards in section 5; and

.2 background information on sampling and analysis methodologies and approaches, set out in the annex.

1.3 Sampling and analysis for compliance testing is a complex issue. According to the *Guidelines for ballast water sampling (G2)*, testing for compliance can be performed in two steps. As a first step, prior to a detailed analysis for compliance, an indicative analysis of ballast water discharge may be undertaken to establish whether a ship is potentially in compliance with the Convention.

1.4 When testing for compliance, the sampling protocol used should result in a representative sample of the whole discharge of the ballast water from any single tank or any combination of tanks being discharged.

2 DEFINITIONS

For the purpose of this guidance, the definitions in the BWM Convention apply and:

.1 A *sample* means a relatively small quantity intended to show what the larger volume of interest is like.

.2 *Representative sampling* reflects the relative concentrations and composition of the populations (organisms and/or chemicals) in the volume of interest. Samples should be taken in accordance with the annex, part 1 and/or part 2 of the *Guidelines on ballast water sampling (G2)*.

.3 *Analysis* means the process of measuring and determining the concentrations and composition of the populations of interest (organisms and/or chemicals) within the sample.

.4 An *indicative analysis* means a compliance test that is a relatively quick indirect or direct measurement of a representative sample of the ballast water volume of interest:

.1 an indirect, indicative analysis may include measurements whose parameters do not provide a value directly comparable to the D-2 standard, including biological, chemical, or physical parameters (e.g. dissolved oxygen levels, residual chlorine levels, Adenosine triphosphate (ATP), nucleic acid, *chlorophyll a*, and that by variable fluorescence, etc. The practicalities, applicability and limitations of these methods should be understood before they are used in compliance testing;

.2 a direct measurement, which is directly comparable to the D-2 standard (i.e. the determination of the number of viable organisms per volume) may also be indicative if it has:

.1 a large confidence interval, or

.2 high-detection limits; and

.3 an indicative analysis is an analysis performed in accordance with sections 4.1 and 4.2.

.5 A *detailed analysis* means a compliance test that is likely to be more complex than indicative analysis and is a direct measurement of a representative sample used to determine the viable organism concentration of a ballast water volume of interest. The result of such measurement:

.1 should provide a direct measurement of viable organism concentration in the ballast water discharge which is directly comparable to the D-2 standard (number of viable organisms per volume);

.2 should be of sufficient quality and quantity to provide a precise measurement of organism concentration (+/- [X] organisms per volume) for the size category(ies) in the D-2 standard being tested for; and

.3 should use a measurement method with an adequate detection limit for the purpose for which it is being applied.

A detailed analysis is an analysis performed in accordance with the methods and approaches in sections 4.3 and 4.4. Detailed analysis should usually be undertaken on a sample taken in accordance with the procedures in section 4.4.

.6 *Testing for compliance* using indicative analysis and detailed analysis can employ a range of general approaches or standard methods. These approaches or methods are divided into those that sample a small proportion of the volume of interest to indicate or confirm compliance or a larger proportion of the volume of interest that can be utilized to indicate and confirm compliance. Those that provide a wide confidence interval should not be used to confirm compliance unless the result and confidence limit are demonstrably over the D-2 standard as measured directly or indirectly. Approaches/Standards are highlighted in sections 4.1, 4.2 and 4.4 for indicative analysis and sections 4.3 and 4.4 for detailed analysis.

.7 *Method* means a detailed step-by-step analysis procedure (for indicative or detailed analysis) or sampling methodology, which the laboratory or organization undertaking the work can follow, be audited against and be accredited to.

.8 *Approach* means a detailed step-by-step analysis procedure (for indicative or detailed analysis) or sampling methodology, which the laboratory or organization undertaking the work can follow. These procedures will not have been validated by an international or national standards organization.

.9 *General approach* means a conceptual description or broad methodology of sample collection or analysis.

.10 *The precision* of a measurement system is the degree to which repeated measurements under unchanged conditions show the same results.

.11 *The detection limit* is the lowest concentration level that can be determined to be statistically different from a blank sample within a stated confidence interval. Limits of detection are method and analysis specific.

.12 *Plankton* means *phytoplankton* (e.g. diatoms or dinoflagellates) and *zooplankton* (e.g. bivalve larvae or copepods) that live in the water column and are incapable of swimming against a current.

.13 *Confidence interval* means a statistical measure of the number of times out of 100 that test results can be expected to be within a specified range. For example, a confidence level of 95% means that the result of an action will probably meet expectations 95% of the time.

.14 *Operational indicator* means a parameter used to monitor and control the operation of the BWMS as defined during testing for Type Approval, e.g. limit values of physical or chemical parameters such as flow rates, dose, etc.

.15 *Performance indicator* means a biological parameter (e.g. ATP, *chlorophyll a*, direct counts) used to estimate or measure the performance of the BWMS in achieving the D-2 standard.

3 PRINCIPLES FOR SAMPLING AND ANALYSIS FOR BALLAST WATER DISCHARGES

3.1 All samples and analysis carried out to determine whether a ship is in compliance with the BWM Convention should be performed under reliable and verified QA/QC procedures (note that any method, approach or sampling procedure should be rigorously validated and practicability should be assessed).

3.2 The first premise of any sampling and/or any analysis protocol is to identify the purpose of the protocol, i.e. to prove whether the discharge of a ship is meeting the D-1 standard or meeting the D-2 standard. There are many ways in which this can be done; however, they are limited by:

.1 the requirements of the methodologies available for sampling the ballast water discharge;

.2 the methods of analysis of samples being collected;

.3 the methods involved in statistically processing the results of these analyses;

.4 the specific operation of the ballast water management system (including when the treatment is applied during the ballast cycle and the type of treatment used); and

.5 the practicalities of sampling a very large volume of water and analysing it for very low concentrations of organisms.

3.3 Successful sampling and analysis is also based on identifying the viable biological population being sampled and its variability. If this population is homogenous, it is much easier to sample than one that is known to be heterogeneous. In the case of ballast water, the sample is drawn from a discharge with a population that can vary significantly. Consequently, the samples collected for indicative or detailed analysis should be representative samples.

3.4 Sampling a ballast water discharge is restricted even further when parts of the ballast water may have already been discharged. Very few inferences can be made on the quality of that ballast water already discharged based on sampling the remaining discharge as it happens. The challenge is to determine the volume of interest and how to sample it.

3.5 The qualitative difference between indicative analysis and detailed analysis often relies on the level of statistical confidence, which, in detailed analysis may be superior.

3.6 Indicative analysis (using operational or performance indicators) can be undertaken at any time throughout the discharge. In cases where indicative analysis identifies that a system is grossly exceeding the D-2 standard, it may be sufficient to establish non-compliance, however, the practicalities, application and limitations of the methodology being used for indicative analysis need to be understood fully.

3.7 Based on the discussion in paragraph 3.3, two different potential detailed sampling approaches can therefore be considered:

.1 sampling the entire discharge from a vessel during a port visit. During this approach:

.1 it will be impossible, by definition, for vessels to discharge prior to sampling;

.2 large numbers of samples are likely to be required over a long period of time;

.3 large sample volumes may be required over a long period of time; and

.4 sampling personnel would be required on the vessel over a significant period of time; and

.2 collecting a representative sample of the ballast water being discharged during some chosen period of time, e.g. one sample or a sequence of samples. During this approach:

.1 the sampling can be developed to fit the situation on board the vessel; and

.2 a representative sample of the discharge can be taken, and that volume can be selected in many ways, providing the opportunity for identifying and sampling specific volumes of the discharge if appropriate, e.g. choosing a percentage of the discharge or sampling duration.

3.8 The D-2 standard expresses a low concentration of organisms to identify in the analysis. The confidence in the result of any sampling and analysis depends on the error inherent in the sampling method and on the error inherent in the method used for analysing the sample. The cumulative error of both must be taken into account when evaluating the result.

3.9 The tables in sections 4.1, 4.2 and 4.3 set out the range of methodologies and approaches, currently identified for use to analyse ballast water discharges and how they relate to the specific sampling protocols in section 4.4. These methodologies and approaches are stand-alone techniques that need to be combined with specific sampling protocols. These protocols should recognize the limitations of each methodology, its inherent sampling requirements, and how it can fit into a comprehensive sampling protocol for compliance testing.

3.10 Although some methodologies and approaches used in type approval testing may also be applicable in compliance testing, the latter, especially indicative sampling, may also require other approaches.

Table 1

Definition and differences between indicative and detailed analysis for the D-2 standard

	Indicative analysis	**Detailed analysis**
Purpose	To provide a quick, rough estimate of the number of viable organisms	To provide a robust, direct measurement of the number of viable organisms
Sampling		
Volume	Small or large depending on specific analysis	Small or large depending on specific analysis
Representative sampling	Yes, representative of volume of interest	Yes, representative of volume of interest
Analysis method		
Analysis parameters	Operational (chemical, physical) and/or performance indicators (biological)	Direct counts (biological)
Time-consuming	Lower	Higher
Required skill	Lower	Higher
Accuracy of numeric organism counts	Poorer	Better
Confidence with respect to D-2	Lower	Higher

4 METHODOLOGIES FOR COMPLIANCE TESTING UNDER THE BWM CONVENTION

4.1 Table 2: Analysis methods that may provide an indication of compliance with the D-1 standard[1]

Indicator	General approach	Standard method	Notes	Level of confidence or detection limit and citation for validation studies
Salinity	Conductivity meter to monitor salinity.	No international standard for ballast water analysis at this time although standard methods for measuring salinity do exist.	External elements can affect the salinity.	To be determined.
Salinity	Refractometer to monitor salinity.	No international standard for ballast water analysis at this time although standard methods for measuring salinity do exist.	Temperature can affect the readings.	To be determined.
Types of organisms in discharge – oceanic, coastal, estuarine or fresh water	Visual identification.	No international standard for ballast water analysis at this time.	Expensive, time-consuming, needs extensively trained personnel; may produce false results if encysted organisms from previous ballasting operations hatch.	To be determined.
Turbidity	Portable turbidity sensors.	No international standard for ballast water analysis at this time.	Requires understanding of turbidity characteristics in relation to the distance from shore.	To be determined.
Dissolved Inorganic and Organic constituents (Nutrients, metals coloured dissolved organic matter (CDOM))	Portable nutrient sensors.	No international standard for ballast water analysis at this time.	Requires understanding of inorganic or organic constituent characteristics in relation to the distance from shore.	To be determined.

[1] Additional information can be found in document BLG 16/4.

4.2 **Table 3: Indicative analysis methods for use when testing for potential compliance with the D-2 standard[2]**

Indicator	General approach	Standard method	Notes	Level of confidence or detection limit and citation for validation studies
Viable organisms ≥ 50 µm	Visual counts or stereo-microscopy.	No international standard for ballast water analysis at this time.	Can be expensive and time-consuming, needs moderately trained personnel. (Note that OECD Test Guideline for Testing of Chemicals 202, "*Daphnia* sp. Acute immobilization test and reproduction test" could be used as basis for standard methodology.)	To be determined.
Viable organisms ≥ 50 µm	Visual inspection.	No international standard for ballast water analysis at this time.	Visual inspection is likely to only register organisms bigger than 1,000 micro-metres in minimum dimension.	To be determined.
Viable organisms ≥ 10 µm and < 50 µm	Variable fluorometry.	No international standard for ballast water analysis at this time.	Only monitors photosynthetic phytoplankton and thus may significantly underestimate other planktonic organisms in this size fraction.	To be determined.
Viable organisms ≥ 50 µm and ≥ 10 µm and < 50 µm	Photometry, nucleic acid, ATP, bulk fluorescein diacetate (FDA), *chlorophyll a.*	No international standard for ballast water analysis at this time.	Semi-quantitative results can be obtained. However, some of these organic compounds can survive for various lengths of time in aqueous solution outside the cell, potentially leading to false positives. Welschmeyer and Maurer (2012).	To be determined.

[2] Additional information can be found in document BLG 15/5/4.

Indicator	General approach	Standard method	Notes	Level of confidence or detection limit and citation for validation studies
Viable organisms ≥ 50 µm and ≥ 10 µm and < 50 µm	Flow cytometry.	No international standard for ballast water analysis at this time.	Very expensive.	To be determined.
Enterococci	Fluorometric diagnostic kit.	No international standard for ballast water analysis at this time.	Minimum incubation time 6 h. Semi-quantitative results from portable methods (see paragraph 2.2.2 of annex 1).	To be determined.
Escherichia coli	Fluorometric diagnostic kit.	No international standard for ballast water analysis at this time.	Minimum incubation time 6 h. Semi-quantitative results from portable methods (see paragraph 2.2.2 of annex 1).	To be determined.
Vibrio cholerae (O1 and O139)	Test kits.	No international standard for ballast water analysis at this time.	Relatively rapid indicative test methods are available.	To be determined.
Viable organisms ≥ 50 µm and ≥ 10 µm and < 50 µm	Pulse counting fluorescein diacetate (FDA).	No international standard for ballast water analysis at this time.	Sampling kit can be larger than that for bulk fluorescein diacetate (FDA).	To be determined.

4.3 **Table 4: Detailed analysis methods for use when testing for compliance with the D-2 standard**

Indicator	General approach	Standard method	IMO citation	Notes	Level of confidence or detection limit and citation for validation studies
Viable organisms ≥ 50 µm and ≥ 10 µm and < 50 µm	Visual counts or stereo-microscopy examination. May be used with vital stains in conjunction with fluorescence + movement.	No international standard for ballast water analysis at this time, but see US EPA ETV Protocol, v. 5.1	BLG 15/5/5 and BLG 15/5/6 BLG 15/INF.6	Can be expensive and time-consuming, needs trained personnel. (Note that OECD Test Guideline for Testing of Chemicals 202, "*Daphnia* sp. Acute immobilization test and reproduction test" could be used as basis for standard methodology.)	To be determined.
Viable organisms ≥ 10 µm and < 50 µm	Visual counts with use of vital stains.	No international standard for ballast water analysis at this time, but see US EPA ETV Protocol, v. 5.1	BLG 15/5/10 (method) BLG 15/5/5 and BLG 15/5/6 (approach) MEPC 58 /INF.10	Requires specific knowledge to operate them. It should be noted that there may be limitations using vital stains with certain technologies.	To be determined. Steinberg et al., 2011
Viable organisms ≥ 10 µm and < 50 µm	Flow cytometers (based on *chlorophyll a* and vital stains).	No international standard for ballast water analysis at this time.	BLG 15/5/5 and BLG 15/5/6	Expensive and require specific knowledge to operate them. It should be noted that there may be limitation using vital stains with certain technologies.	To be determined

Indicator	General approach	Standard method	IMO citation	Notes	Level of confidence or detection limit and citation for validation studies
Viable organisms ≥ 50 µm and Viable organisms ≥ 10 µm and < 50 µm	Flow cameras (based on *chlorophyll a* and vital stains).	No international standard for ballast water analysis at this time.	BLG 15/5/5 and BLG 15/5/6	Expensive and require specific knowledge to operate them. It should be noted that there may be limitations using vital stains with certain ballast water management systems.	To be determined
Viable organisms ≥ 50 µm and Viable organisms ≥ 10 µm and < 50 µm	Culture methods for recovery, regrowth and maturation.	No international standard for ballast water analysis at this time.	BLG 15/5/5 and BLG 15/5/6	Require specific knowledge to conduct them. Densities are expressed as Most Probable Numbers (the MPN method). Most species do not manage to grow using this method therefore cannot be used alone. 2-3 weeks incubation time needed.	To be determined
Enterococci	Culture methods.	ISO 7899-1 or ISO 7899-2	BLG 15/5/5 and BLG 15/5/6	Requires specific knowledge to conduct them. At least 44-h incubation time. EPA Standard Method 9230	To be determined.
Escherichia coli	Culture methods.	ISO 9308-3 or ISO 9308-1	BLG 15/5/5 and BLG 15/5/6	Requires specific knowledge to conduct them. At least 24-h incubation time. EPA Standard Method 9213D	To be determined.

Indicator	General approach	Standard method	IMO citation	Notes	Level of confidence or detection limit and citation for validation studies
Vibrio cholerae (O1 and O139)	Culture and molecular biological or fluorescence methods.	ISO/TS 21872-1/13/	BLG 15/5/5 and BLG 15/5/6	Requires specific knowledge to conduct them. 24-48 h incubation time. US EPA ETV Fykse et al., 2012 (semi-quantitative pass/fail-test) Samples should only be cultured in a specialized laboratory.	To be determined.
Enterococci, *Escherichia coli, Vibrio cholerae (O1 and O139)*	Culture with 11holera11ence-in-situ hybridization (FISH)	No international standard for ballast water analysis at this time.		Requires specific knowledge to conduct them. Quantitative and qualitative results after 8 h. Samples should only be cultured in a specialized laboratory.	To be determined.
Viable organisms ≥ 50 µm and viable organisms ≥ 10 µm and < 50 µm	Visual counts using stereo-microscopy examination and flow cytometry.	No international Standard for ballast water analysis at this time.	BLG 17/INF.15	A Sampling Protocol that identifies whether a system is broken or not working and producing a discharge that is significantly above the D-2 standard. Designed to detect gross non-compliance with 99.9% confidence. Needs to be Validated.	To be determined.

4.4 **Table 5: General approaches for sampling use when testing for compliance with the BWM Convention**

General approaches for sampling	Discharge line or BW tank	Citation for validation study or use	Sample error and detection limit	Relative sample error amongst approaches
Filter skid + isokinetic sampling	Discharge line	Drake et al., 201First et al., 2012 (land-based testing); shipboard validation underway, Prototype 01, SGS	To be determined	Lower
Cylinder containing plankton net + isokinetic sampling	Discharge line	MEPC 57/INF.17	To be determined	Lower
Sampling tub containing plankton net + isokinetic sampling	Discharge line	Gollasch, 2006 and Gollasch et al., 2007 Cangelosi et al., 2011	To be determined	Lower
Continuous drip sampler + isokinetic sampling	Discharge line	Gollasch and David, 2010, 2013	To be determined	Lower
Grab sample	BW tank	David and Perkovic, 2004; David et al. 2007, BLG14/INF.6	To be determined	Higher

4.5 **Table 6: Sampling and analysis methods/approaches for use when testing compliance with the BWM Convention. A checkmark indicates an appropriate combination of sampling and analysis.**

Analysis type size class or indicator microbe analysis method/approach	**Filter skid + isokinetic sampling**[3]	**Plankton net + isokinetic sampling**	**Continuous drip sampler + isokinetic sampling**	**Grab sample**
Indicative Analysis ≥ 50 µm Visual inspection Stereomicroscopy counts Flow cytometry Nucleic acid ATP *Chlorophyll a,* Bulk *FDA*	✓	✓		
Indicative Analysis < 50 µm and ≥ 10 µm variable fluorometry Flow cytometry Nucleic acid ATP *Chlorophyll a,* bulkBulk *FDA*			✓	✓

[3] Methods other than using an isokinetic approach as defined in Guidelines (G2) for acquiring a representative sample may be used in certain circumstances. Such methods should be validated prior to use.

Analysis type size class or indicator microbe analysis method/approach	**Filter skid + isokinetic sampling**[3]	**Plankton net + isokinetic sampling**	**Continuous drip sampler + isokinetic sampling**	**Grab sample**
Indicative Analysis Enterococci, *E. coli* Fluorometric diagnostics			✓	✓
Indicative Analysis *Vibrio 14holera* Test kits Culture methods + microscopy			✓	✓
Detailed Analysis ≥ 50 µm Stereomicroscopy counts Flow cytometry/Flow camera	✓	✓		
Detailed Analysis < 50 µm and ≥ 10 µm Visual counts + vital stain(s) Flow cytometry/Flow camera Culture methods			✓	
Detailed Analysis Enterococci, *E. coli* Culture methods FISH with pre-cultivation			✓	
Detailed Analysis *Vibrio 14holera* Culture methods FISH with pre-cultivation			✓	

4.6 References

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ANNEX 2

TECHNICAL DISCUSSION FOR THE GUIDANCE TO BALLAST WATER SAMPLING AND ANALYSIS IN ACCORDANCE WITH THE BWM CONVENTION AND GUIDELINES (G2)

1 INTRODUCTION

1.1 The purpose of this annex is to provide background information on:

.1 the development and use of methodologies for both indicative and detailed analysis and appropriate sampling; and

.2 analysis of the sample at an accredited laboratory.

1.2 This annex highlights the advantages, disadvantages and limitations of many different measures. Although recommendations are given in this document on what methodologies may be used, there are distinct benefits in using certain technologies at certain times. This should not stop the use of any of the methodologies, as long as the limitations are taken into account.

1.3 Any methods for analysis used for assessing compliance with the BWM Convention should be carefully validated under a range of operating conditions.

2 INDICATIVE ANALYSIS: METHODOLOGY AND APPROACHES

2.1 The D-1 standard

2.1.1 The D-1 standard requires the vessel to exchange its ballast water 200 nm from the coastline in waters 200 m deep, or if this cannot be achieved for safety reasons, 50 nm from the coastline in waters of the same depth. Therefore, the water in exchanged ballast water should have a similar salinity to that of mid-ocean water.

2.1.2 Indicative analysis for the D-1 standard of the BWM Convention could rely on the chemical parameters (e.g. salinity) of the water in the ballast water discharge, or on an estimate of species present. However, the latter might need trained personnel. If the ballast water discharge being tested has a salinity significantly less than that of 30 PSU, then it is likely that the ballast water has not been exchanged en route under the conditions required in the D-1 standard, or that the exchange has not been completed successfully.

2.1.3 Two exceptions to this are:

.1 when ballast water is taken up in port areas that are located in high-salinity environments, above 30 PSU. In such a case ballast water with a PSU of 30 may not originate from mid-ocean waters and therefore the ship may not be compliant with the D-1 standard; or

.2 when ballast water has been exchanged in designated ballast water exchange areas within 50 nm from the coastline in waters that may be of less salinity than the mid-ocean water. In this case the ballast water exchange would be compliant.

Therefore, the origin of the last ballast water exchange should be known before interpreting the results of salinity analysis.

2.1.4 Checking salinity could be backed up by further analysis of the organisms in the ballast water discharge to determine the origin of the ballast water; however, this would take time and need experienced staff. This can be done in line with the visual analysis methodologies outlined in paragraph 2.4.3 below. However, it should be noted that there are many external factors that could affect the salinity and the organisms in the ballast water, such as wet sediments in the ballast tanks, the state of the tide in the port concerned during its uptake and the fact that exchange may not remove all coastal organisms.

2.1.5 There are many ways to quickly and easily monitor the salinity of water on the market, and generic salinity measures should be used for indicative analysis.

2.2 Bacteria levels in the D-2 standard

2.2.1 Bacterial levels could be tested by a wealth of available portable methods. However, as the D-2 standard for bacteria is measured in colony forming units (CFU), the systems utilized may have to include a specific incubation time of the samples, which for commercially available systems is never shorter than four hours. Therefore, the time it takes for incubation limits the use of such systems for indicative analysis.

2.2.2 Advances in fluorometric diagnostics have resulted in a methodology that identifies the presence or absence of bacteria in a sample of the ballast water discharge. This methodology is based upon the detection of enzymes produced by the target bacteria in unconcentrated fresh water or marine samples and presently easily portable test kits for E. coli and Enterococci are available. This method can identify low levels of bacteria in water samples in less than 10 minutes, but the results are only semi-quantitative, i.e. a low level reading equates to a low level of bacteria. However, although the presence of bacteria can be shown, whether or not these organisms are living (i.e. form colonies) cannot be proven with this method at the present time. These diagnostic methods could be used in indicative analysis if very large numbers of organisms are identified.

2.3 Organisms of less than 50 micrometres and greater than or equal to 10 micrometres in minimum dimension[4] in the D-2 standard

2.3.1 Methods to measure the organisms in this category of the D-2 standard can be divided into two categories as follows:

.1 the use of biological indicators for organisms:

.1 nucleic acid;

.2 adenosine triphosphate (ATP), a coenzyme used as the main energy storage and transfer molecule in the cells of all known organisms; and

.3 indicators for the presence of organisms, such as *chlorophyll a*;

.2 the use of direct counts of living organisms (coupling a means to determine viability and manual or automatic counting of individual organisms).

[4] The "Minimum Dimension" means the minimum dimension of an organism based upon the dimensions of that organism's body, ignoring e.g. the size of spines, flagellae or antenna. The minimum dimension should therefore be the smallest part of the "body", i.e. the smallest dimension between main body surfaces of an individual when looked at from all perspectives. For spherical shaped organisms, the minimum dimension should be the spherical diameter. For colony forming species, the individual should be measured as it is the smallest unit able to reproduce that needs to be tested in viability tests. This should be considered whenever size is discussed in this document.

2.3.2 The presence of nucleic acid or ATP in a sample may be taken as an indication of life, but it should be noted that this nucleic acid or ATP could come from any living organism of any size within the sample. There are no definitive methods available to correlate the amount of nucleic acid or ATP with the amount, or viability of organisms in the sample and, therefore, the presence of these chemicals are limited as an indicative analysis methodology. However, zero measurements of these chemicals may indicate that no organisms are in the sample, i.e. the treatment process was successful and in the D-2 standard is being met. Additionally, if nested filters are used to isolate specific size groups, then ATP, which degrades relatively quickly, can provide an indication of the potential presence of a large concentration of organisms in one size class. If linked to thresholds of ATP concentrations, this can be used to indicate samples which are highly likely to be above the standard.

2.3.3 The same problems occur when using other bio-chemical indicators to monitor the number of organisms in this category. As many of the organisms in this size range are likely to be phytoplankton, an obvious step would be to measure the level of *chlorophyll a*, a photosynthetic pigment which is essential for photosynthesis in the sample. Zero concentrations may indicate that there is no phytoplankton in the sample and chlorophyll *a* may also be a good indicator as to whether a BWMS using an oxidizing process was working to design dosages, as it might be expected to bleach such pigments. However, caution has to be exercised as:

.1 *chlorophyll a* can persist in seawater outside of a cell, therefore, sampling should only be limited to the particulate phase. However, nucleic acid and ATP can exist in dead organisms, detrital material, senescent or dead cells, decomposing macroalgae, plant detritus from terrestrial ecosystems and other non-living particles, etc.;

.2 there may be zooplankton in the sample being analysed;

.3 no cell count can be directly measured from a *chlorophyll a* measurement, as many small cells may provide a similar signal strength to that of fewer bigger cells; and

.4 no size distinction can be made and the *chlorophyll a* could derive from phytoplankton in the larger size category of the D-2 standard.

As a consequence, direct concentration measurements of this chemical would be difficult to use in indicative analysis. A wealth of portable tools exists to document the *chlorophyll* a content in seawater.

2.3.4 One potential exception is the Pulse-Amplitude Modulated Fluorometer (PAM) which measures the *chlorophyll a* fluorescence in living cells by exciting *chlorophyll a* molecules and registering the subsequent fluorescent signal. Such a response is only available in living cells and it should be noted that this method only provides an indirect measurement of those phytoplankton that use *chlorophyll a* in the sample, in both size categories of the D-2 standard. Testing this methodology on ballast water discharges suggests that there is a correlation between the ratio of variable and maximum fluorescence and the number of phytoplankton in this size category. However, the relationship between fluorescence signals and mixed assemblages of phytoplankton from different locations needs to be validated.

2.3.5 For analysis of organisms above 10 microns in minimum dimension, a flow cytometer may also be used. A common element of these systems is that they automatically count objects, including organisms, per size class in a fluid. The more simplified systems cannot separate organisms from sediment and detritus, or living from dead organisms. More

sophisticated systems can also assess organism viability for phytoplankton by using organism stains together with flow cytometry. The separation of living phytoplankton from detrital material and zooplankton is based on the presence of auto chlorophyll fluorescence of phytoplankton cells. It should be noted, however, that using *chlorophyll a* fluorescence as an indicator of living organisms may result in over counting, as the molecule can remain intact for a significant amount of time as has been proved in preparing fixed (dead) samples. The practicability to use such devices on board a ship should be carefully assessed before use. To make a stable stream to produce adequate size of water particles, the device should be set in perfectly horizontal. Also any vibration should be isolated for accurate measurement.

2.3.6 Systems using flow cytometry deliver automated results promptly and may be used to assess the number of living phytoplankton in a sample after treatment with a viability stain. However, readings provided by the flow cytometer should also be examined manually to verify the automated readings. Concerns have been raised by users that the viability of smaller algae may not always be categorized correctly in these systems, as the viability signal may be too low for detection. Other concerns include the efficiency of portable versions and the limited ability of some of them to monitor organisms greater than or equal to 50 micrometres in minimum dimension. Although these systems may become a major tool in the future, there are elements, such as the reliability of portable versions of the systems that limit their use at the present time, which is especially the case for organisms greater than or equal to 50 micrometres in minimum dimension. Also, it is not clear if the time to analyse a sample is greater than can be allotted in compliance testing. These can be overcome by taking the sample off the ship and using a fixed or mobile system near to the ship or the port.

2.3.7 Visual inspection could be another method of indicative analysis that is a quick and simple way to justify the need for detailed analysis. Taking an appropriate sample, concentrating it if necessary, and visually inspecting it against the light may show living organisms in the sample, but it should be noted that without magnification a visual inspection is likely to result in only organisms greater than or equal to 1,000 micrometres in minimum dimension being detected, unless chains or clumps are formed by colony forming organisms or the density of organisms is sufficiently large to colour the water. An assessment of the viability in such an inspection is limited to complete body movements of the organisms as organ activity and antennae or flagella movements may not be seen. As samples from BWMS that are not compliant are likely to contain organism levels that are orders of magnitude above the D-2 performance standard, visual inspections could be used in indicative analysis. However, it is assumed that only organisms bigger than 1,000 micrometres in minimum dimension may be determined in such way, therefore, its use for this size category is limited.

2.3.8 Visual inspection can also be undertaken using a field stereomicroscope with a low magnification (e.g. x 10). However, this methodology may require concentration of the sample and may need analysis by a trained operator to detect viable organisms. It should also be noted that this methodology would be more efficient and practicable for organisms greater than or equal to 50 micrometres in minimum dimension.

2.4 Organisms greater than or equal to 50 micrometres in minimum dimension in the D-2 standard

2.4.1 Many of the methodologies for monitoring organisms less than 50 micrometres and greater than or equal to 10 micrometres in minimum dimension may also be valid for monitoring organism levels in this category. However, nucleic acid and ATP methodologies encounter the same problems as outlined in paragraphs 2.3.2 and 2.3.3; and monitoring *chlorophyll a* levels, through fluorometers or the PAM methodology described above, has limited value for this size category of the D-2 standard, as the majority of organisms in this category are likely to be zooplankton.

2.4.2 Visual inspections may significantly underestimate the number of organisms in this size category due to the issues described in paragraph 2.3.8. However, the method may be robust enough to determine whether the BWMS is working at orders of magnitude above the D-2 standard based on a simple extrapolation from the sample to the D-2 standard. Detailed analysis may be needed to confirm this, especially when levels near the D-2 standard are encountered.

2.4.3 Additionally, stereomicroscopy can also be used to identify viable organisms greater than or equal to 50 micrometres in minimum dimension. The sample should be concentrated appropriately. Viability assessment should be based on movements of intact organisms. This movement may be stimulated. In addition, organ activity should be observed and fully intact non-moving organisms which show organ activity should be counted as living. Stains might also be used to help in viability determination – though methods are still under development. The viable organism numbers should be recorded and the numbers extrapolated up to the total volume of water filtered.

2.4.4 If the results in paragraphs 2.4.2 and 2.4.3 show elevated levels of organisms, then this result will indicate that the D-2 standard is not being met.

2.4.5 Further research must be encouraged; innovative methods for assessing for D-2 compliance, preferably based on in situ, automatic sampling and analytical procedures, should facilitate the most uniform implementation of the BWM Convention.

2.5 Operational indicators

Other indirect parameters and indicators could be used to indicate whether a BWMS is meeting the D-2 standard. These include, but are not limited to, indicators from the electronic self-monitoring of the BWMS and residual chemicals (or lack of) from the BWMS, such as dissolved oxygen levels, residual chlorine, etc.

3 DETAILED ANALYSIS METHODOLOGIES AND APPROACHES

Once detailed analysis has been instigated by the port State, they should be prepared to undertake full analysis of the sample at an appropriate laboratory.

3.2 Bacteria

3.2.1 There are already international standards in place to analyse for the bacteriological indicators contained within the D-2 standard.

3.2.2 For Enterococci, ISO 7899-1 or 7899-2; or Standard Method 9230 (in the United States) should be used, and ISO 9308-3, ISO 9308-1 or Standard Method 9213D (in the United States) are appropriate for Escherichia coli. The methods used should be quantitative and based on a 95-percentile statistical evaluation. The number of laboratory samples should be sufficient to define the mean and standard deviation of Log 10 bacterial enumerations.

3.2.3 For *Vibrio cholerae* ISO/TS 21872-1/13 is appropriate. 100 ml of ballast water should be filtered and incubated according to ISO/TS 21872-1. Analysis needs to be undertaken in a specialist laboratory.

3.3 Organisms of less than 50 micrometres and greater than or equal to 10 micrometres in minimum dimension

3.3.1 Many of the analysis methods used to ascertain the numbers of organisms within this category have already been discussed in section 2. However, section 2 focuses on indicative analysis, rather than the more detailed analysis. Therefore, the following sections examine these methodologies in more detail. Some of these methodologies discussed here also relate to organisms greater than or equal to 50 micrometres in minimum dimension.

3.3.2 Simple upright and inverted microscopes are very useful for the enumeration of morphologically healthy organisms and motile organisms, as well as for measuring the size of organisms. Using this technology needs some skill and experience to evaluate the health of the individual organisms in the sample. However, this technology and experience should be available globally.

3.3.3 Fluorescence generated from photosynthetic pigments can be used for more detailed analysis of the morphological health of organisms and for the evaluation of stained organisms and a microscope with fluorescence capabilities is needed. However, this methodology only identifies phytoplankton (both living and dead) in the sample and makes no size differentiation. Zooplankton should be analysed through the methods highlighted in section 3.4.

3.3.4 Fluorescein di-acetate (FDA), chloromethylfluorescein diacetate (CMFDA) and Calcein-AM vital stains have both been used to determine viability. When non-specific esterases (enzymes found in live cells) are present, they cleave the acetate groups from the stains, and the resultant fluorescein molecules fluoresce green when illuminated with a blue light from an epi-fluorescence microscope. This method works best with live samples. Microscopes with a fluorescence capability and operators with skills and experience of analysis should be available at universities and research laboratories worldwide. However, it should be noted that these stains do not always work on all species or at all salinities and further research to validate this approach may be needed to support the use of these stains for this type of analysis.

3.3.5 Flow cytometers are advanced technologies which can be used in a laboratory to determine size, and viability of organisms in ballast water when a reliable vital stain(s) is (are) used to indicate organism viability. Cytometer detected particles, including organisms, can be processed visually or by a computer to quantify viable organisms in that sample. These systems reduce manual labour, but require specific knowledge to operate them. High particle loads in ballast water may reduce the detection limits of these methodologies and the volume of samples analysed. At present, portable versions of these technologies have not fully been proven for use on ballast water discharges, however, samples could be taken off the ship and analysed using a fixed or mobile system near to the ship or the port.

3.3.6 Regrowth experiments, in which the visual appearance of photosynthetic organisms in a sample is followed by a specific period in order to quantify the Most Probable Number (MPN), are methods to evaluate the number of organisms in a sample. However, these are slow and are work intensive. In addition, a major drawback of this methodology may be that specific growth factors during the incubation may not be fulfilled, giving a risk of bias. Regrowth and reproduction may be seasonably variable, giving different results at different times. Further, a viable organism may be in good health and reproducing rapidly, or in poor health, not reproducing until health has improved. Finally, this is likely to be time-consuming.

3.3.7 Bulk parameter measurements, such as photosynthetic activity, are also not suitable for detailed analysis (please see paragraphs 2.3.2 and 2.3.3), but can be used as supporting data for other methods used to determine the number of viable organisms in the ballast water samples.

3.3.8 Planktonic organisms may be fragile and samples may need to be concentrated further to aid the accurate quantification of organisms. There are many methods to achieve this, however, care has to be taken to reduce physical stress as this may result in reduced viability levels. A simple, rapid, flexible and cautious method for concentrating plankton cells is the use of transparent membrane filters. If the sample analysis is performed on board the sample can be filtered directly on to this membrane, which can subsequently be placed directly under a microscope for examination. The sample volume to be analysed would need to be adjusted depending on the cell density, however, live, vital stained and fixed organisms within this size category can be evaluated on these filters. If the representative analysis is performed at a laboratory, this process for concentration should be performed at the laboratory just before starting the staining process to avoid under-estimate of viable organisms. Importantly, the loss (if any) of organisms (i.e. those cells passing through the filter and recovered in the filtrate) would need to be determined. Alternatively, a filter mesh may be used to concentrate the sample and the concentrated organisms may, after filtration, be transferred into an observation chamber. Again, the loss of organisms through damage must be quantified.

3.4 Organisms greater than or equal to 50 micrometres in minimum dimension in the D-2 standard

3.4.1 Paragraphs 3.3.2 to 3.3.8 are also applicable to the analysis of organisms in this size category.

3.4.2 In addition, the following issues need to be considered when developing a methodology for analysing organism numbers in this size category:

.1 testing the sample for movement and response to different stimuli are simple techniques for the examination of viable/dead zooplankton under a stereomicroscope. The observation for organ activity, such as heartbeats, may also contribute to the viability assessment. The use of a filtering mesh (e.g. 50 microns in diagonal dimension) under the Petri dish of the stereomicroscope, or the addition of 50 micron micro beads to the sample, may help with size calculations and vital stains may also add value to these methodologies. Separate guidelines on this issue are being developed through the land-based facilities and the ETV protocol in the United States;

.2 methods using a combination of flow cytometry and microscopy have the disadvantage of high complexity, high price and small sample sizes, which means the ballast water samples would have to be concentrated further; and

.3 the storage condition and time before analysis is likely to be critical to reduce mortality in the sample.

3.4.3 It is therefore recommended that simple microscopic examination of organisms in this size category is used for compliance monitoring. The microscopic examination of organisms is a robust, simple and cheap methodology which can be completed in laboratories worldwide.

4 SOURCES OF ERROR

4.1 The ideal method for compliance monitoring is a procedure that:

.1 detects organisms in the ballast water discharge;

.2 has an appropriate limit of detection;

.3 is precise;

.4 is accurate;

.5 is economical;

.6 is quick;

.7 can be carried out with minimal technical expertise; and

.8 can be obtained in all parts of the world.

However, any result obtained would have to include confidence limits based on both the sampling error and analytical error.

4.2 Sources of error include, but are not limited to, errors arising within:

.1 sampling, including:

.1 sample loss (e.g. during filtration);

.2 incorrect use of equipment;

.3 day-to-day variations in the conditions in which the sampling is taking place; and

.4 the experience of the technicians;

.2 processing the sample, including:

.1 incorrect use of equipment;

.2 day-to-day variations in the conditions in which the sampling is taking place; and

.3 the experience [and fatigue] of the technicians;

.3 analysis of the sample:

.1 incorrect use of equipment;

.2 the experience [and fatigue] of the technicians;

.3 day-to-day variations in the conditions in which the sampling is taking place;

.4 the number of organisms counted. The distribution of organisms in a range of samples usually follows the Poisson distribution and higher numbers of samples give a lower relative variation and sample error;

.5 the inherent variation and errors arising from the methods used for analysis. This is especially so when the evaluation of organism numbers in a sample is based on manual counting methods due to human error. For example, although the definition of the minimum dimension of an organism in Guidelines (G2) is quite detailed,

analytical results may be influenced by practical issues. These include situations when the size of an organism is determined on a two dimensional microscope, which cannot view the organism "from all perspectives"; and

.6 poor harmonization between laboratories and quality control within the laboratory. In the field of chemical analysis, inter-laboratory calibration occurs and is tested. Inter-laboratory calibration of biological samples is also common practice, but the difficulty in the compliance monitoring context is that the viability of the organisms needs to be documented and the viability may be impaired by the mode and duration of sample shipments to different laboratories. Therefore, laboratories should be well managed, and uncertainty limits (the analysis variation) should be calculated for each laboratory. This should be achieved in conjunction with ISO 17025, which provides a standard for the general requirements needed by laboratories to prove they are competent to carry out tests and/or calibrations, including sampling.

4.3 The variation arising from sampling should be added to that from analysis to determine the confidence limits within which the true value of the organism number lies. This has an important bearing on how the result can be used for enforcement of the BWM Convention.

4.4 The sampling uncertainty can be obtained by setting up a null–hypothesis, that is a general or default position that is expected in the results, e.g. the average concentration of organisms is equal to the D-2 standard at a selected level of significance and then the data would be analysed using one of the following tests:

Table 1: Statistical handling of the results

Distribution of the results	Test	Notes
Normal distribution	t-test	It is unlikely this test will be used, as it is not used with "rare" populations, i.e. the expected population of organisms in treated ballast water
A distribution that is not normal	Non-parametric Wilcoxon rank test	Not normal due to the small number of samples
Poisson distribution	Chi-square test	Used when the analytical results are treated as one sample (i.e. the numbers of organisms over the entire volume are very rare [low] and combined).

Ideally, an analysis of the distribution should be performed before the data are statistically evaluated.

4.5 There has been much discussion within IMO on whether the results of the analysis should be averaged to assess compliance or that every result should have to meet the D-2 standard. This is a unique debate at IMO due to the biological nature of the subject

matter being analysed, and different States have significantly different views on this issue. Therefore, it will be very difficult to arrive at a conclusion as in the case of non-compliance the results of the analysis are likely to be used in the legal jurisdictions of each IMO Member State, and each of those States may require different evidence to support any enforcement action.

4.6 If the results of detailed analysis are to be averaged, then both the sample variation and the analysis variation need to be calculated and applied to the result. However, some analysis of the sample variation may be needed, as it may be unacceptably high. For example, for five treated ballast water samples, viable organism number results of 9,9,9,9 and 9 will provide the same average as 0,0,0,0 and 45. Both systems would pass the D-2 standard, if averaged; however, the variation is considerably bigger for the second set of results and may prove to be unacceptable because of the one large value.

4.7 If each of the results is treated as an individual value that has to meet the D-2 standard, then again the confidence limits would have to be calculated from the sampling and analytical errors. Here if all results are less than the D-2 standard, then the sampling has proved that the BWMS is meeting the standard.

4.8 The basic difference between instantaneous and average approaches is that the results of the average approach describe the variations of the concentration of organisms during the deballasting event, whereas the results of the instantaneous approach describes the variation based on the assumptions of the Poisson distribution. However, the average approach, based on the results of a few samples, has the disadvantage that the variation may be too high, is unacceptable and needs to be improved, which could invalidate the evaluation and lead to inconclusive results.

4.9 The instantaneous approach has the disadvantage that variations in the organism levels at different times of the discharge are not taken into account, which should not be a problem if all the samples meet the D-2 standard. If the discharge is not always under the D-2 standard, the problem can be mitigated by using a flow-integrated sample over set periods of time, which, if taken properly, represents an average of the organisms in the treated ballast water over that time when presented with variance estimates and confidence intervals. This constitutes a better representation of the ballast water quality than separate samples. In addition, a lower variation should be obtained because a larger sample is being analysed. The average approach is likely to have the same disadvantages unless the samples are very large and collected over most of the discharge.

4.10 The differences between applying an instantaneous sampling regime or an average sampling regime to the result are less extreme when taking numerous flow-integrated samples. This is because for each discharge there will be a number of results arising from samples that have been averaged over a specific time.

5 DETAILED ANALYSIS: THE SAMPLE PROTOCOL

5.1 Sample protocols for discharges of treated ballast water through a distinct discharge point fall into two categories, the first based on specified and replicated volumes and the second based on flow integration over a specified time. The first entails taking a specific number of set volumes of the ballast water discharge, whilst the second takes a continuous sample over a set time period. The flow integration sampling protocol can be achieved by either continuously sub-sampling a small amount throughout the entire duration of the discharge, therefore, collecting one sample over time, or taking multiple sub-samples over a specific time scale (i.e. 5 minutes, 10 minutes or 15 minutes) repeatedly throughout the discharge, providing a result for each sub-sample.

5.2 However, for sampling protocols based on specified and replicated volumes, defining both the number of samples and their volume to ensure representativeness, takes time. As a representative sampling procedure is needed to ensure compliance with the BWM Convention, then the flow integration protocols based on set times should be implemented.

5.3 Using a sampling protocol that continuously sub-samples small amounts throughout the entire duration of the discharge, may significantly underestimate the amount of larger organisms (i.e. organisms greater than or equal to 50 micrometres in minimum dimension) in the sample due to damage to the organisms held in the cod-end of the filter. If such a system is used then a protocol for replacing the cod end needs to be developed.

5.4 The arrangements for detailed analysis should take into account the requirements of the methods and/or approaches they intend to use for detailed and/or indicative analysis. Special consideration should be given and contingencies arranged for sampling in remote ports, where it is likely to take time to mobilize samplers and sampling resources.

6 DETAILED METHODOLOGY

6.1 As described in paragraph 5.1, there are two distinct ballast water sampling protocols, one based on flow integration and one based on the use of specified and replicated volumes. As they both use filtration and concentration of the sample the following section can apply to both methods.

6.2 For in-line sampling, a sampling system should be set up which:

.1 collects organisms greater or equal to 50 µm;

.2 allows samples of the ballast water to be taken and filtered;

.3 enables the amount of ballast water sampled to be measured to allow for extrapolation of the results; and

.4 allows the filtered ballast water to be discharged safely without affecting the stability and safety of the ship, its crew and the samplers or other discharges from the vessel such as bilge water.