Summary and Findings of the Ballast Discharge Monitoring Device Workshop

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Prepared by Allegra Cangelosi and Nicole Mays

NORTHEAST MIDWEST INSTITUTE

218 D Street SE ▪ Washington, DC 20003 ▪ 202/544-5200 ▪ www.nemw.org

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218 D St, SE
Washington DC 20003
Ph. 202 544 5200
www.nemw.org

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ABSTRACT

Aquatic invasive species are a leading threat to marine biodiversity. Ballast water of commercial vessels is the major pathway by which aquatic species are distributed globally. Both national and international law will in time mandate that ships undertake some form of ballast water management to attenuate organism transfers. Ballast water management could take the form of operational practices, such as high seas ballast water exchange (BWE); ballast water treatment (BWT), such as physical separation or biocides; or a combination of the two. Development of such a standard has been slow due to many technical issues, especially the best approach to expression of a standard. Debate revolves around potential protectiveness, and sampling efficiency. One consideration that has not been thoroughly explored is the extent to which analytical tools exist to support evaluations against proposed standards. The form in which the standard is expressed will dictate in many ways specific analytical requirements both for treatment evaluations (e.g. for regulatory approvals) and enforcement monitoring (e.g. for real time detection of a problem). Do we currently have the tools to analyze ballast discharge consistent with the approaches to standard expression currently under discussion? How far are we from having them? And what will be necessary to usher in their development? An international Ballast Discharge Monitoring Device Workshop was convened to explore the answers to these questions. The meeting took place at the U.S. Geological Survey's Western Fisheries Research Center's Marrowstone Island Field Station on the Olympic Peninsula, Washington from August 12th - 16th, 2002. The goal of the Workshop was to assess existing and emerging analytical tools (technologies and techniques) for their potential to support regulatory evaluations of ballast discharge associated with the range of standards under discussion. In addition, the group identified characteristics of the "ideal" discharge evaluation system and recommended research objectives that could make the ideal a reality.

WORKSHOP PARTICIPANTS

Chip Blatchley
Purdue University
Indiana, USA
blatch@ecn.purdue.edu

Allegra Cangelosi
Northeast-Midwest Institute
Washington DC, USA
acangelo@nemw.org

Jonathon Champion
Northeast-Midwest Institute
Washington DC, USA
jchampion@nemw.org

Jeff Cordell
University of Washington
Seattle, Washington, USA
jcordell@u.washington.edu

Flavio Fernandes
Admiral Paulo Moreira Marine
Research Institute
Brazil
flaviocofe@yahoo.com

Russ Herwig
University of Washington
Seattle, Washington, USA
herwig@u.washington.edu

Jose Matheickal
Nanyang Technological University
Singapore
jmatheic@imo.org

Nicole Mays
Northeast-Midwest Institute
Washington DC, USA
nmays@nemw.org

Tracy McCollin
Fisheries Research Services
Scotland
T.A.McCollin@marlab.ac.uk

Ben Paulson
University of Washington
Seattle, Washington, USA
billie36@u.washington.edu

Jake Perrins
University of Washington
Seattle, Washington, USA

Frank Shipley
Western Fisheries Research Center
Seattle, Washington, USA
frank_shipley@usgs.gov

Mike Taylor
Cawthron Institute
New Zealand
michael@cawthron.org.nz

Nick Welshmeyer
Moss Landing Marine Laboratories
California, USA
welschmeyer@mlml.calstate.edu

Dana Woodruff
Battelle - Pacific Northwest National Laboratory
Washington, USA
Dana.Woodruff@pnl.gov

David Wright
Chesapeake Biological Laboratory
Solomons, Maryland, USA
wright@cbl.umces.edu

BACKGROUND AND PROBLEM STATEMENT

Regulations are now under development at the state, national and international level to prevent ship-mediated transfers of invasive species. Most of them target ballast water as the primary mode for species transfers by ships, and call for high-seas ballast water exchange (BWE), and eventually, ballast water treatment (BWT) to reduce the probability of ballast-transfers of unwanted organisms. BWT is considered more promising than BWE in the long term because BWE is limited in its effectiveness, scope of safe application and enforceability.

Neither management method (BWE or BWT) comes equipped with a ready approach to assessing effectiveness or monitoring. BWE is currently monitored using one of two indirect methods. The "Newcastle Verification Method" evaluates ships' log information and uses algorithms involving the vessel's speed, pump rate, and energy consumption during exchanges (Taylor, 2004). The U.S. Coast Guard measures salinity of ships' ballast water visiting the Great Lakes as a surrogate measure for BWE. Both methods are considered inadequate regulatory tools. Meanwhile, BWT is not yet required, so no monitoring method is in current use.

A performance standard for ballast management methods, both BWE and BWT, could stimulate regulation, and investment in development of better approaches but only if the standard is both meaningful and measurable (Northeast-Midwest Institute, 2001; Royal Haskoning, 2001). The two fundamental approaches to performance standards that have been discussed are standards which require: 1) a minimum process efficiency expressed as percent or log reductions relative to intake or control concentrations of organisms/near coastal water; and 2) a minimum discharge quality, such as maximum discharge concentrations of organisms. The first approach is being employed by the U.S. Coast Guard to guide its determinations under the Shipboard Technology Evaluation Program (U.S. Coast Guard, 2004), and by Congressional sponsors of the National Aquatic Invasive Species Act of 2003 (S. 525, H.R. 1080/1081) to guide BWT approvals during an interim regulatory period in which ships have the option of either treating or exchanging ballast water. A percent/log reduction in live aquatic organisms relative to intake or control parallels the current approach to expressing effectiveness of BWE, which replaces a percentage of harbor water (and some lower percentage of entrained organisms) with open ocean water.

The second approach to performance standards -- a maximum allowable concentration of organisms in ballast discharge – applies primarily to BWT, and has been advocated for use in formal rule-making by the maritime industry and federal agencies. It was also selected by the International Maritime Organization (IMO) for the standard within its recent "International Convention for the Control and Management of Ship's Ballast Water and Sediments" (IMO, 2004). In the 108th Congress, Senators introduced legislation (S. 2490) that would codify a version of the IMO standard in U.S. law. This approach forces the treatment system to control for geographic and seasonal variation in intake density. That is, the system must be capable of treating the entire range of potential intake densities to meet the same discharge standard.

Most of the discussion around these two approaches to a performance standard for ballast management has revolved around the questions of protectiveness and practicability. In terms of protectiveness, everything depends upon one's definition of success. To the extent that the goal of BWT is to improve protection relative to untreated ballast or relative to BWE, a percentage

approach could facilitate comparisons. Such an approach may be particularly relevant in an "interim" regulatory period or during technology development, when the goal is to significantly improve on the status quo. However, a percent/log reduction approach is a relativistic one; allowable concentrations of organisms at discharge, though less than intake/control densities, would vary with intake concentrations. Indeed, discharge concentrations on some voyages could exceed intake concentrations on others. Such a standard, therefore, will not reflect a given level of environmental protectiveness.

The second approach to a standard offers the prospect of limiting discharge to a particular level by a given ship. If science could identify what discharge level would be protective given the number of ships and volumes of ballast they may discharge into a given harbor, a truly protective standard could be derived. However, uncertainty over "how clean is clean" remains a technical obstacle to this goal. To begin to address this question, researchers must characterize the nature and condition of biological constituents in ballast discharge over time for a range of harbors, and attempt to link this information with predictions of invasion risk of the receiving systems. This work will take some time. Meanwhile, a working group of the International Council for the Exploration of the Sea (ICES) recommended that a standard be established guaranteeing that discharges will contain at least three orders of magnitude fewer live organisms than the mean of untreated discharge globally for each major taxonomic grouping (ICES, 2003). In a sense this compromise position hybridizes the logic behind both the percent/log approach and discharge limit approach. A three log reduction is sought, but rather than looking at control and treatment estimates on a ship by ship basis, the three log reduction is sought relative to a fixed estimate for the entire global fleet.

At least in the near term, while uncertainty around protective discharge levels reins, practicality, or the ability to implement (monitor and enforce against) the standard, is a more urgent consideration than effectiveness. Most discussion around this question has considered the logistics of obtaining samples for analysis, and the extent to which the samples could be taken directly from the ship. Requiring all ships to treat ballast so that live organism densities are a given percent/log lower in treated discharge than untreated or intake densities is most feasible using a time-limited type approval procedure involving known intake and discharge quality. Such a type approval scenario could be accompanied by shipboard spot-checks for verification. However, this sort of standard could also be enforced using in-line taps in the ballast line to take before/after or with/without treatment samples concurrently. Either way, two sets of samples would be needed for each measurement. For this reason, it makes sense from a policy standpoint to use this approach in instances in which the numbers of vessels requiring monitoring is limited. Accordingly, this approach was selected by the U.S. Coast Guard to guide its determinations under the Shipboard Technology Evaluation Program (U.S. Coast Guard, 2004), and by Congressional sponsors of the National Aquatic Invasive Species Act of 2003 (S. 525, H.R. 1080/1081) to guide treatment approvals during an interim regulatory period in which ships have the option of either treating or exchanging ballast water.

Meanwhile, the second approach to expression of a standard could be implemented more easily directly at the ship because it would require sampling only the treated discharge stream. Organism densities in intake and control streams would be irrelevant. This approach offers apparent efficiencies and a more direct measure of compliance. In deciding upon a standard, the

IMO altered upward the ICES-recommended limits for plankton, and added limits for specific pathogenic microbes in ballast discharge, but retained the approach of setting absolute discharge limits based on an estimate of untreated discharge densities of the global fleet.

Unfortunately, the implementation debate has not adequately taken into account the state of science for analyzing ballast water samples consistent with the standards discussed, though enforcement against each approach to standard expression requires a distinct set of analytical capabilities. To defensibly detect and enumerate viable or live organisms in an unknown assemblage across the taxonomic spectrum found in harbor water around the globe is a monumental analytical undertaking. Where the topic has been raised, there has been little consensus. The IMO used concentration limits in ballast discharge for an array of size and taxonomic categories on the assumption that analytical tools are or will become available. While the U.S. supported this approach to a standard, it advocated for substitution of the term "live" rather than "viable" for detection purposes. Mann (2004) has testified before Congress that a size cut-off for live organisms would facilitate detection through use of a metabolic dye test. Others have argued that ATP analysis could be used as a detection procedure (Waite *et al*., 2003).

In order for any standard to be effective at stopping the transfer of invasive species, it will need to be supported by analytical methods to enforce and verify compliance. This report provides a summary of a workshop held to explore the range of analytical tools available to characterize ballast water discharge and their possible applications to ballast water regulatory objectives.

WORKSHOP PURPOSE

Under a cooperative agreement with the U.S. Geological Survey's Western Fisheries Research Center (WFRC), the Northeast-Midwest Institute convened an international Ballast Discharge Monitoring Device Workshop at the WFRC's Marrowstone Island Field Station on the Olympic Peninsula, Washington from August 12th - 16th, 2002. The purpose of the Workshop was to explore and describe the state-of-the-art in tools currently suitable, potentially available, and ideally available for analyzing ballast water biota in support of a range of treatment/management standards. It is hoped that this information will assist policy-makers and regulators to understand the extent to which analytical tools may support the various types of standards and regulatory activities relevant to ballast water discharge regulation.

METHOD

Workshop participants were assembled based on their expertise with analytical methods and ballast management issues. Participants included scientists and subject experts from the U.S., Brazil, New Zealand, Singapore and the United Kingdom, and scientific instrument vendors, including Beckman Coulter Inc., Fluid Imaging Technologies and Meridian Instrument Co. At the Workshop, participants identified the array of possible standard-types under discussion and regulatory objectives relevant to regulation of ballast management systems. Next, they identified the analytical needs associated with these applications, both functional and operational. They

spent a great deal of time identifying and assessing analytical tools that could be used to meet these needs now or at some point in the future. Finally, based on this information, participants assessed the current and potential analytical capacity to approve, monitor and enforce against the range of standards currently under discussion for ballast discharge regulation.

1. REVIEW CRITERIA

The suite of necessary functional capabilities varies with the particular regulatory objectives, including the subject practice (BWE or BWT), the subject taxonomic group (zooplankton, including pelagic life stages of crustaceans, mollusks, and echinoderm; phytoplankton, including cysts and unicellular taxa; and bacteria), the regulatory activity (type approval versus enforcement monitoring), and type of standard (percent/log reduction versus discharge limit or discharge prohibition of particular live or viable organisms). To assess relevancy of each analytical tool to ballast water regulatory assessments, the participants developed a matrix consisting of 1) possible types of standards; 2) types of regulatory activities associated with each standard; 3) functional needs associated with each regulatory activity relative to each standard; 4) operational considerations; and 5) future potential.

1.1. Types of Standards

To review the tools for their potential applicability to ballast-related regulatory activities, participants evaluated analytical requirements for regulation of BWE and BWT separately, and identified the following possible types of standards for the two operations. It should be noted that some types of standard lend themselves to the entire suite of taxa while others do not, and actual regulatory proposals therefore have combined these approaches.

- ***BWE – Prescribed Physical Dilution of Ballast Water with Mid-Oceanic Water***

This approach is proposed in pending national legislation, the National Aquatic Invasive Species Act of 2003 (S. 525, H.R. 1080/1081) which requires ships to operate pumps long enough to achieve 95 percent purge of near coastal water with any BWE. The IMO's International Convention for the Control and Management of Ship's Ballast Water and Sediments requires a purge equivalent to three tank volumes or fewer, if fewer can achieve a 95 percent purge. Final U.S. Coast Guard regulations (Federal Register Vol. 69, No. 144, July 28, 2004) require a volume equal to three tank volumes or one complete empty/refill with no mention of percent purge. These performance criteria are not directly related to changes in organism density.

- ***BWE – Prescribed Biological Dilution of Ballast Tank Biota with Mid-Oceanic Biota***

This approach to a BWE standard is not currently in use, but is a conceivable way to link BWE to biological performance. Regulatory agencies could require a minimum percent reduction in some indicator coastal planktonic organisms -- or an increase in some indicator high seas planktonic organisms -- for any BWE to be considered complete.

- ***BWE - Presence/Absence Based on Trigger Concentrations of Biological Indicators of Near Coastal Water***

The third option is purely theoretical, but at times discussed. It is to spot-check incoming ballast water for the presence of unwanted near coastal target species (such as red tide, or *Vibrio cholera*), and attach consequences to ships in which these organisms are found to exist.

- ***BWT - Percent or Log Reductions of Live/Viable Organisms Relative to Control or Intake Levels***

This approach is applied in the interim standard in the state of Washington program, which requires a 95 percent kill or removal of zooplankton and 99 percent kill or removal of phytoplankton and bacteria, and the standard for the initial regulatory period (during which BWE is still acceptable) in the National Aquatic Invasive Species Act of 2003 (S. 525, H.R. 1080/1081), which required a 95 percent reduction in zooplankton and phytoplankton relative to intake. The approach is also applied in the U.S. Coast Guard's Shipboard Technology Evaluation Program for experimental treatment evaluation (U.S. Coast Guard, 2004).

- ***BWT - No Detectable Live/Viable Organisms above a Certain Size Limit***

This approach was listed as an option in the U.S. Coast Guard's September 26, 2003 Federal Register notice, recommended in Congressional hearings, and at the IMO GloBallast Standards Workshop. It is presumed that this approach would not be applied to bacteria.

- ***BWT - Limited Density of Live/Viable Organisms within Size Limits or Within Taxa***

This approach was listed as an option in the U.S. Coast Guard's September 26, 2003 Federal Register notice, the IMO's International Convention for the Control and Management of Ship's Ballast Water and Sediments, and legislation in the 108th Congress, S. 2490. The ICES Ballast Working Group also recommended this approach using taxonomic groups rather than size categories (ICES, 2003).

1.2. Types of Regulatory Activities Associated with Each Standard

In addition, participants identified two separate regulatory activities associated with these subject activities and standards:

- ***Shipboard Spot-Checks (both BWE and BWT)***

Routine monitoring against a standard during ship operations to detect any gross divergence from expected performance along the lines of a pass-fail test. A shipboard spot-check can be less comprehensive than verification or type approval testing.

- ***Shore-Based Treatment Verification and Type Approval (primarily BWT)***

One-time intensive assessment of treatment system performance under controlled challenge conditions against a standard. This testing would likely be more comprehensive and longer-term than spot-checks, and able to detect gradations of performance.

1.3. Functional Capabilities Associated with Each Application

Participants then made assumptions regarding the types of functional capabilities that would be needed for each regulatory objective and the range of possible standards, and arrayed them by taxonomic group, *independent of the actual availability of tools which could deliver them*. In general, the analytical functions considered most critical to biological evaluations of ballast water were those that could deliver relative to zooplankton, phytoplankton and bacteria, including:

- Taxonomic identification
  - Presence/absence of specific organisms/taxa
- Differentiation of organism condition
  - Viability (ability to reproduce)
  - Live/dead status
- Organism size
- Organism enumeration
- Bulk measurements of total or viable biomass

However, the specific suite of relevant functions will vary with the type of standard and regulatory activity. Table 1 summarizes the Workshop participants' view of the suite of capabilities associated with ballast water evaluations for each possible type of BWE and BWT standard, and for the three major taxonomic groups (zooplankton, phytoplankton and bacteria).

1.4. Operational Capabilities Associated with Each Application

Analytical tools used for ballast water spot-checks must be user-friendly as the measurements will take place in the field at a variety of locations. Ideally, they:

- Do not require extensive sample preparation or care.
- Can be carried out in the field by relatively untrained technicians.
- Are portable.
- Are robust enough to withstand a harsh shipboard environment.
- Provide results in a reasonable period of time.
- Are low maintenance.
- Are relatively inexpensive.
- Can process large volumes of sample relatively quickly

Tools used in shore-based verifications and approval scenarios can be more involved and require greater training and expense, but their results should be readily replicable and not be susceptible to heavy operator bias.

Table 1. Analytical needs by major taxonomic group for each possible type of BWE and BWT standard. (Z = zooplankton, P = phytoplankton, B = bacteria, * = within taxa only, ** = within size limits only)

	Approach	Standard Type	Taxonomic Identification	Absolute Density	Viability of individuals	Relative density/ biomass of viable population	Size
BWE	Process efficiency	Prescribed Physical dilution					
	Process efficiency	Prescribed Biological dilution	Z, P	Z, P			
	Discharge quality	Presence of biological indicators	Z, P, B	Z, P, B			
BWT	Process efficiency	Percent/log reduction in live organisms				Z, P, B	
	Discharge quality	No detectable live organisms including above a size limit			Z, P, B		Z, P, B
	Discharge quality	Limited density of live organisms within size limits or within taxa	Z*, P*, B*	Z, P, B*	Z, P, B*		Z**, P**

1.5. Future Potential

The potential for the analytical tools to have improved relevancy in the future given methods development or technological enhancement was also considered. The time and effort that developing the tools to be more relevant might require was linked to the likelihood that the improvement could be achieved.

2. TOOLS REVIEWED

Any analytical method delivering information relevant to analysis of zooplankton, phytoplankton and bacteria, including pathogens, was considered potentially relevant to regulatory objectives associated with ballast management. The participants identified the following categories of analytical tools as being the best prospects for ballast water analysis. Though there is some overlap among these categories, they represent fundamentally distinct approaches to analysis.

- **Particle Counting and Sizing**
  - Optical sensing and sizing
  - Electrical sensing particle counting and sizing

- **Fluorescence Detection for Organism Counting and/or Sorting**
  - Flow cytometry using fluorescence-activated cell sorters
  - Flow cytometry using fluorescent dyes
  - High performance liquid chromatography

- **Visual Organism Counting, Sorting and Sizing**
  - Conventional microscopy
  - Microscope/camera
  - Optical zooplankton counting

- **Hybrid Methods for Counting, Sorting and Sizing**
  - Imaging flow cytometry

- **Molecular Detection Methods**
  - Polymerase chain reaction
  - Quantitative polymerase chain reaction
  - Laser scanning cytometry
  - Matrix assisted laser detection ionization mass spectroscopy

- **Biochemical Viability Assays**
  - Electron transport system assay
  - Adenosine tri-phosphate assay
  - Chlorophyll *a* extraction
  - Phytoplankton stress/death enzyme

- **Biochemical/Physical Matrix Assays**
  - Physical dye assay
  - Multivariant chemical/physical assay

REVIEW OUTCOME BY ANALYTICAL TOOL

The output of the review process is presented below by analytical tool. For each tool, there is a general description, an assessment of present relevancy to each regulatory objective given functional and operational considerations, and a discussion of possible future relevancy.

1. PARTICLE COUNTING AND SIZING SYSTEMS

1.1. Description

Particle counting and sizing systems automate a complex particle analysis of an aquatic matrix, irrespective of particle type. Two fundamental approaches to particle counting and sizing are available:

- ***1.1.1. Optical Sensing Counting and Sizing Systems (e.g., AccuSizer 780/APS Automatic Particle Sizer by Particle Sizing Systems)***

Optical particle counting and sizing uses a narrow, uniform beam of light to analyze particles suspended in liquid as they pass through an illuminated "photozone". The passage of the particle through the photozone produces a detected pulse, the magnitude of which depends on the mean diameter of the particle and the physical principle of detection -- light scattering or obscuration

(blockage). The example system (fig. 1) incorporates a patented Autodilution system, where the sample is automatically diluted to the optimal concentration. The particle suspension medium is made sufficiently dilute that the particles pass, one at a time, through the illuminated region, avoiding coincidences. An illumination/detection system in the sensor is designed to provide a monotopic increase in pulse height with increasing particle diameter. A particle size distribution is constructed one particle at a time by comparing the detected pulse heights with a standard calibration curve obtained from a set of uniform particles of known diameter. The system is capable of sizing particles across a wide distribution of sizes (0.5 to 2,500 microns) with resolution and accuracy. Samples are analyzed at a rate of 60 – 180 mL/minute. Output of the analysis is available within 2 – 3 minutes using Microsoft compatible programs. See www.pssnicomp.com for more detailed information.

Fig, 1. AccuSizer 780/APS Automatic Particle Sizer by Particle Sizing Systems

- ***1.1.2. Electrical Sensing Particle Counting and Sizing Systems (e.g., Multisizer 3 Coulter Counter by Beckman Coulter)***

This particle counting and sizing approach utilizes the Coulter Principle to measure particle volume -- a direct measurement of a physical property of the particle. Here, particles suspended in a weak electrolyte solution are drawn through a small aperture separating two electrodes between which an electric current flows. The voltage applied across the aperture creates a "sensing zone". As each particle passes through the aperture (or "sensing zone") it displaces its own volume of conducting liquid, momentarily increasing the impedance of the aperture. The change in impedance produces a tiny but proportional current flow into an amplifier that converts the current fluctuation into a voltage pulse large enough to be measured accurately. The amplitude of this pulse is directly proportional to the volume of the particle that produced it (Coulter Principle). Scaling these pulse heights in volume units using interchangeable orifice tubes of different sizes ranging 20 to 2,000 microns, enables a size distribution to be acquired and displayed. In addition, if a metering device is used to draw a known volume of the particle suspension through the aperture, a count of the number of pulses will yield the concentration of particles in the sample. The example system (fig. 2) is capable of measuring particles from 0.4 to 1,200 microns and automatically corrects for coincidence. It analyzes samples ranging in volumes of 50 µL to 2000 µL. Output of the analysis is available within seconds using Microsoft compatible programs. See www.beckman.com for more detailed information.

Fig. 2. Multisizer 3 Coulter Counter by Beckman Coulter

1.2. Functional and Operational Fit (Present)

- ***1.2.1. Functional***

Particle counting and sizing systems can achieve a range of functions relative to ballast water analysis, including:

- ✓ Sort particles into size bins to determine overall size distribution.
- ✓ Enumerate large numbers of particles in a size range.
- ✓ Yield highly comparable results from one application to the next.

However, several limitations constrain the usefulness of this type of detection technology for ballast discharge analysis. For example, particle sizing systems cannot:

- ✕ Differentiate types of particles (including animate or inanimate).
- ✕ Differentiate metabolic condition of organisms (e.g., live/dead).
- ✕ Indicate particle dimensions, detecting instead particle "volume" which is then translated into size dimensions of a sphere with that volume.
- ✕ Provide measurements of bulk viable biomass.

Table 2 summarizes the functional capabilities of particle counting and sizing systems for each major taxonomic group.

Table 2. Functional capabilities of particle counting and sizing systems by major taxonomic group

	Zooplankton	Phytoplankton	Bacteria
Taxonomic identification			
Organism condition			
Organism size	Potential	Potential	Potential
Organism enumeration	Potential	Potential	Potential
Bulk measurements of viable biomass			

- ***1.2.2. Operational***

Particle counting and sizing systems typically require only moderate sample preparation. Apertures need to be changed during analysis if a wide size range is desired. Training for proper use of these methods is somewhat demanding. However, technicians require no scientific qualifications. Portable systems could be useful onboard vessels, but there are concerns over durability and electromagnetic interference. Systems are also moderately expensive.

Ballast water discharge analysis could also create special challenges for particle counting and sizing systems. Though organisms may represent a high percentage of particles in upper size bins (100 microns or more) in ballast water, they are extremely sparse relative to particles in lower size bins (clay and silt). The autodilution function helps with dense samples, but not sparse ones. It will likely be necessary for the operator to also concentrate samples to achieve statistically meaningful numbers. Concentrating samples for purposes of particle counting introduces error and uncertainty as clumping can occur during the concentration process.

- ***1.2.3. Conclusion***

In light of these functional and operational considerations, particle counting and sizing systems could provide a means for limited shipboard spot-checking, but their greatest utility is in support of land-based type approval studies. Specific potential applications include:

- ✓ Shore-based BWT verification or type approval for determining changes in numbers in a pure culture of an indicator organism (percent reduction standard), and changes in numbers in a pure culture of an indicator organism following a grow-out period (for verification against viability standards).
- ✓ Spot-checking BWT (physical separation devices only) for evaluation against a size-based standard to determine particle removal capacity irrespective of particle type or condition (but if size is characterized as a minimum dimension, then particle counters will not be adequate).

Given the capabilities and limitations of particle counting and sizing systems, the systems are currently not well suited to:

- ✕ Shipboard monitoring against a BWT standard expressed as numbers of live organisms per volume of ballast water or a size cut-off (cannot distinguish organism viability or organisms from other particles).

1.3. Prospects and Timeliness for Improved Relevancy

Methods development and automation for effectively concentrating samples would improve the reliability of this approach, and therefore its utility in the limited applications cited above. Over time, particle counting and sizing systems could be applied to:

- ✓ Spot-checking BWE to determine if a large population of a target organism (e.g., coastal organism) in a particular size range is present or absent.

The ability to make this extrapolation, however, would be limited to organisms that occur in distinctive "blooms", and which are not in the same size category as inanimate particles. An example could be dinoflagellates. In the end, other methods also may be best for this purpose (such as molecular detection methods). The systems, by themselves, also cannot achieve other uses, except through the use of dyes and fluorescence described below as a separate category of technology.

Table 3 summarizes the current and potential regulatory applications of particle counting and sizing systems for each type of BWE and BWT discharge standard.

Table 3. Current and potential regulatory applications of particle counting and sizing systems for each type of BWE and BWT discharge standard

	Type of Standard	Shore-Based Verification/ Type Approval	Shipboard Spot-Checks
BWE	Physical dilution		
	Biological dilution		
	Presence biological indicators		Potential
BWT	Percent/log reduction in live organisms	Yes	
	No detectable live organisms above a size limit		Yes
	Limited density of live organisms (above a size limit or within taxa)	Yes	

2. FLUORESCENCE DETECTION FOR ORGANISM COUNTING AND/OR SORTING

2.1. Description

Fluorescence detection measures the energy expressed by various biochemicals upon excitation by a laser beam. Three fundamental approaches to fluorescence detection are available:

- ***2.1.1. Flow Cytometry Using Fluorescence-Activated Cell Sorters (e.g., BD FACS Calibur Flow Cytometer by BD Biosciences)***

This fluorescence detection approach utilizes a laser beam and continuous flow of a fine stream of suspension medium to separate and measure cells. Each cell scatters some of the laser light, and also emits fluorescent light excited by the laser. Several parameters can simultaneously be measured for each cell, including low angle forward scatter intensity (proportional to cell diameter), orthogonal scatter intensity (proportional to the quantity of granular structures within the cell), and fluorescence intensities at several wavelengths. The example system (fig. 3) is the only four-color, dual laser, benchtop system currently available that is capable of both cell analysis and sorting. It is designed to support a wide range of applications and is fully integrated and multi-parameter. It combines dual-laser technology, an automated sample loader option for 12 x 75-mm tubes, and Microsoft-compatible software. It can also sort cells directly onto filters or cell culture inserts. It is capable of analyzing samples at three different flow rates (60 µL/minute, 35 µL/minute, and 12 µL/minute) with a sample concentration of 10^5 to 2 x 10^7 particles/mL. Cells are sorted at a rate of 300 cells/minute. See www.bdbiosciences.com for more detailed information.

Fig. 3. BD FACS Calibur Flow Cytometer by BD Biosciences

- ***2.1.2. Flow Cytometry Using Fluorescent Dyes***

Though flow cytometry is a powerful and versatile technique, it is not itself a dependable means of distinguishing among types of particles of the same size, or the metabolic condition of organisms (live/dead), and the success of the analysis, even for simple numbers of organisms irrespective of condition, is entirely dependent on the sample preparation. The use of fluorescent

dyes is one way to prepare the sample to yield the greatest possible amount of information. Many different dyes may be used depending on the sample type and parameters being measured. Generally, the dyes work by binding to a variety of cytochemical components. The main criteria for the fluorescent dyes' applicability to flow cytometry is the excitation wavelength -- it must match the available wavelength of the light source. The procedure of fluorescent dye labeling is determined primarily by the protocol required of the dye being used. Different types of dyes of use include fluorescein and tetramethylrhodamine (for general fluorescent labeling), DAPI (to measure size and nucleic acid content of bacteria), and SYTOX-Green (to stain algal cells that have lost their membrane integrity).

- ***2.1.3. High Performance Liquid Chromatography (HPLC)***

Pigment analysis with HPLC is a rapid, very sensitive and reliable method for determining phytoplankton composition and estimating the biomass of specific algal groups. An HPLC system (fig. 4) separates substances according to their relative adhesion to a "stationary phase" (a solid substance) and a "mobile phase" (a liquid or gas flowing past the stationary phase). The crux of HPLC is a column, which consists of tightly packed plastic beads (the stationary phase). The sample of pigment dissolved in an appropriate solvent (the mobile phase) is pumped through the column. As the mobile phase passes through the column, different photosynthetic pigments are retarded to different degrees according to their hydrophobicity and hydrophilicity. A detector measures the pigments as they come off the column according to their absorbance or optical density at a pre-set wavelength (or wavelengths). The different pigments emerge from the column one at a time rather than all at once, resulting in separate peaks on the output. The detector automatically measures the area of each peak; this area can then be related to pigment concentration through a series of separate calibration steps. Systems are capable of analyzing volumes at a rate of 1 mL/minute or faster. Information is provided using Microsoft-compatible software. For more information visit http://hplc.chem.shu.edu/HPLC/index.html.

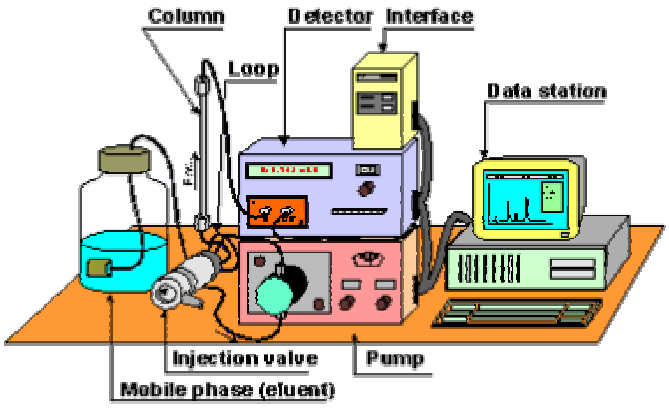

Fig. 4. Functional schematic of HPLC system (courtesy http://hplc.chem.shu.edu)

2.2. Functional and Operational Fit (Present)

- ***2.2.1. Functional***

Fluorescence detection systems (flow cytometers and HPLC) offer some advantages for ballast discharge analysis over particle counting and sizing systems in that they are capable of a slightly greater degree of particle characterization. The characterizations are still quite rough, however, such that these methods will add value only when it is useful to seek a distinctive taxonomic grouping of organisms within an assemblage. It may be possible to use these methods, for example, to distinguish types of phytoplankton on the basis of fluorescence and light-scatter characteristics.

In summary, fluorescence detection systems can do everything that optical counting and sizing equipment can do, plus:

- ✓ Distinguish organisms from inanimate particles.
- ✓ Distinguish broad taxonomic groupings (e.g. phytoplankton from zooplankton) within an assemblage.
- ✓ Determine phytoplankton composition and estimate the biomass of specific algal groups (HPLC only).
- ✓ Distinguish live/dead condition of any organisms for which there is a reliable metabolic dye (e.g. bacteria).
- ✓ Estimate viability based on counts of pure cultures of organisms before and after a grow-out period.

Limitations that constrain the usefulness of this type of detection technology for ballast analysis include inability to:

- ✕ Reliably distinguish between live and dead organisms in an unknown assemblage (especially plankton).
- ✕ Reliably distinguish taxonomic groupings of organisms in an unknown assemblage.

Table 4 summarizes the functional capabilities of fluorescence detection systems for each major taxonomic group.

Table 4. Functional capabilities of fluorescence detection systems by major taxonomic group

	Zooplankton	Phytoplankton	Bacteria
Taxonomic identification		Potential	
Organism condition		Potential	Potential
Organism size	Yes	Yes	Yes
Organism enumeration	Yes	Yes	Yes
Bulk measurements of viable biomass		Potential	

- ***2.2.2. Operational***

Though fluorescence detection systems require only moderate sample preparation, training is needed to collect and process samples. Sample analysis, even using analysis software, also requires substantial scientific training, and the process is somewhat slow. The systems may also be too delicate and/or expensive for use onboard vessels; though, portable flow cytometers are under development for use in a shipboard application.

- ***2.2.3. Conclusion***

Fluorescence detection systems could be used in combination with another technology (e.g. image analysis software) to determine organism counts by taxonomic group, as well as bulk counts. The application of these methods to BWT evaluation and verification situations is somewhat limited, however, due to a lack of reliable species-specific stains and antibodies. Systems can be of use immediately for:

- ✓ Shore-based BWT verification or type approval analysis for determining total numbers (live plus dead) and changes in numbers after grow-out (viability) of spiked zooplankton, phytoplankton and bacteria (in context of any standard type).
- ✓ Shipboard BWT spot-checks for determining reductions in bulk Chlorophyll *a* and/or numbers of phytoplankton cells relative to intake or control (only useful in context of a percentage reduction standard)

2.3. Prospects and Timeliness for Improved Relevancy

Reliable metabolic stains for particular taxonomic groups could enhance the utility of flow-cytometers for BWT type approval and verification exercises on land. In addition, tools such as HPLC could resolve and automate determinations of community composition (e.g., dinoflagellates, diatoms, etc.), possibly in support of shipboard spot-checks of BWE. The ability to use such methods for spot-checking BWT compliance against any of the standards except possibly the percent reduction standard is not promising because live/dead analysis will be limited to only those taxonomic groups for which effective stains have been developed.

Over time, fluorescence detection systems could be applied to:

- ✓ Shipboard BWE spot-checks determining changes in numbers of indicator organisms, or coastal species of zooplankton, phytoplankton and/or bacteria (biological standard type).
- ✓ Shipboard BWT spot-checks for determining integrity of physical separation devices through particle counting and sizing capability.

Table 5 summarizes the current and potential regulatory applications of fluorescence detection systems for each type of BWE and BWT discharge standard.

Table 5. Current and potential regulatory applications of fluorescence detection systems for each type of BWE and BWT discharge standard

	Type of Standard	Shore-Based Verification/ Type Approval	Shipboard Spot-Checks
BWE	Physical dilution		
	Biological dilution		Potential
	Presence biological indicators		Potential
BWT	Percent/log reduction in live organisms	Yes	Yes
	No detectable live organisms above a size limit	Yes	
	Limited density of live organisms (above a size limit or within taxa)	Yes	Potential

3. VISUAL METHODS FOR ORGANISM COUNTING, SORTING AND SIZING

3.1. Description

With visual methods, the human eye and magnification are employed to conduct assays. Three fundamental approaches to visual detection are available:

- ***3.1.1. Conventional Microscopy***

This visual method of counting, sorting and sizing involves the application of microscopes and counting chambers to identify and enumerate bacteria, phytoplankton and zooplankton. Stereo microscopes (fig. 5) are the most common systems used and can magnify objects 2 – 540 times. Stereo microscopes provide a reliable method of counting, sorting and sizing zooplankton, phytoplankton, and colony forming units of bacteria. Concentrated samples (in some cases facilitated by live/dead stains) can also be analyzed using this approach to provide precise information on numbers of live organisms. In contrast, compound microscopy is a much more powerful form of microscopy and can magnify objects 600 – 1,000 times using an oil interface between lens and slide. This approach is typically used to investigate cellular structure and function. Other approaches to microscopy include epifluorescent microscopy (a type of microscopy that uses light of an appropriate wavelength to stimulate fluorescence in samples of bacteria and phytoplankton) and electron microscopy (a type of microscopy that produces high-resolution images by the interaction of electrons with the specimen). Electron microscopy is capable of magnifying specimens in excess of 250,000 times, with a resolution of less than 1 nm. Visit www.microscopyu.com for more information.

Fig. 5. Nikon Stereo Microscope

- ***3.1.2. Microscope/Camera* (e.g., *Nikon Digital Net Camera DN100 and Microscope)***

This visual method of counting, sorting and sizing uses a platform independent system featuring a 1.3 megapixel color CCD camera capable of capturing images at a rate of 15 frames per second. A primary component of the system is the camera control unit. This unit allows the microscopist to digitally process, save, and manipulate digital images on a stand-alone basis, over a local area network, or over the Internet. The control unit digitally processes an input video signal transmitted by the CCD camera or an external input line, and produces an output video signal with a maximum pixel resolution of 1280 x 960. The example system (fig. 6) is also equipped with a platform-independent networking capability that includes communications based on either HTTP or FTP. This feature allows multiple users to simultaneously connect to the DN100 camera control unit and to assess still images, video stream, and images saved to a PC card or the FTP server housed in the instrument. For best results, samples must be stationary when capturing still images, or moved slowly for short video clips. The optical power of the attached microscope determines the size and resolution of the specimen, and corresponding digital image. Visit www.microscopyu.com for more information.

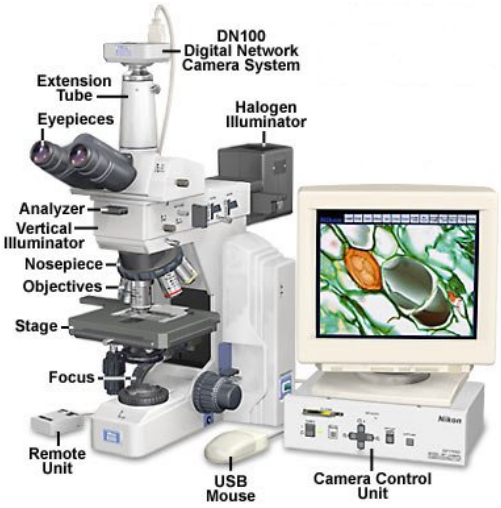

Fig. 6. Nikon Digital Net Camera DN100 and Microscope

- ***3.1.3. Optical Zooplankton Counting***

This visual approach to counting, sorting and sizing is useful for providing both bench top and in-situ real-time data on numbers and sizes of zooplankton in considerably less time than conventional methods. The technology uses a parallel light beam to form a sensing zone whereby a digital size unit proportional to the amount of light blocked by the individual organism is registered for each zooplankter passing through the sensing zone (fig. 7). The system records raw data in thousands of size categories and then automatically converts it to equivalent circular diameters based on calibrations and linear equations. Bench top and underwater systems are effective in measuring plankton with diameters from 0.25 to 20 mm. Bench top units use either a submersible pump or circulation system to introduce samples into the system in a controlled manner. Underwater remote units can be towed at speeds of up to 12 knots and used in depths of 1000 m. Visit www.focaltech.ns.ca/product-opc.html for more information.

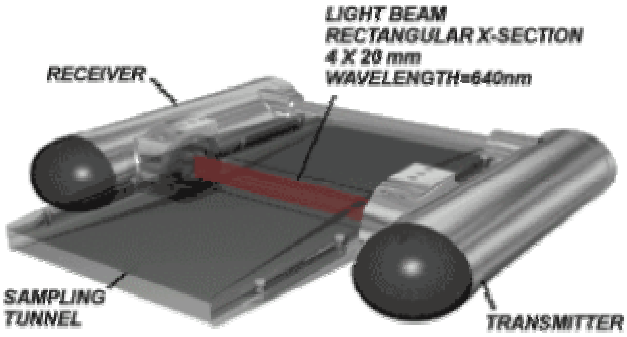

Fig. 7. Optical Zooplankton Counter by Focal Technologies Corporation

3.2. Functional and Operational Fit (Present)

- ***3.2.1. Functional***

Visual methods can achieve a wide range of functions relevant to ballast water analysis, including:

- ✓ Provide accurate counts, sizes and species identifications in concentrated ballast water samples for a broad range of taxa.
- ✓ Distinguish live and dead zooplankton.
- ✓ Estimate viability based on before/after grow-out comparisons for culturable bacteria, phytoplankton and zooplankton.

In terms of limitations, visual methods cannot:

- ✕ Discern live/dead phytoplankton or viable/inviable bacteria, phytoplankton or zooplankton without a grow-out arrangement.

✕ Conduct live analysis on zooplankton without operator bias.

Table 6 summarizes the functional capabilities of visual methods of counting, sorting and sizing for each major taxonomic group.

Table 6. Functional capabilities of visual methods by major taxonomic group

	Zooplankton	Phytoplankton	Bacteria
Taxonomic identification	Yes	Yes	Yes
Organism condition	Yes	Yes	Yes
Organism size	Yes	Yes	Yes
Organism enumeration	Yes	Yes	Yes
Bulk measurements of viable biomass			

- ***3.2.2. Operational***

Live/dead analysis, counting and sorting of organisms microscopically is an extremely time consuming and labor-intensive process. Visual methods of counting, sorting and sizing require a highly trained technician to collect, concentrate, conduct live analysis, stain and/or preserve the samples. Live analysis must be also completed within one hour of collection. Numerous individual organisms must be evaluated in order to assure statistical power of findings. Microscope cameras could allow less trained individuals to collect and record sample contents, but analysis is limited to enumeration (as opposed to live/dead), and still requires a trained taxonomist to interpret the results. Microscopes are suited to use onboard vessels if they are secured properly, and are not disturbed by vessel movements and vibrations. Systems are also moderately expensive, but require little maintenance.

- ***3.2.3. Conclusion***

Visual methods, particularly conventional microscopy, will play an important role in analysis for live zooplankton, plankton community composition, and the enumeration of colony forming units of bacteria in support of:

- ✓ Shore-based verification/type approval against all BWT standards.
- ✓ Shipboard spot-checks against all types of BWT standards.
- ✓ Shipboard spot-checks of BWE effectiveness, including through use of remote camera systems, by distinguishing high seas and near coastal indicator assemblages.
- ✓ Ground-truthing the effectiveness of more hybrid methods for counting, sorting and sizing plankton.

3.3. Prospects and Timeliness for Improved Relevancy

Visual methods for counting, sorting and sizing remain the best analytical tools currently available to assess ballast discharge composition and quality. Use of this method for live/dead analysis would be greatly facilitated by reliable and broad spectrum metabolic stains. Samples could even be preserved and counted later. Such stains could also make visual approaches such as microscope cameras workable tools for ballast analysis. However such stains are not likely to be reliable except for selected taxa. As such, the efficiencies afforded by stains could be limited to shore-based applications in which stainable sentinel species are spiked into the ballast flow.

Table 7 summarizes the current and potential regulatory applications of visual methods of counting, sorting and sizing for each type of BWE and BWT discharge standard.

Table 7. Current and potential regulatory applications of visual methods for each type of BWE and BWT discharge standard

	Type of Standard	Shore-Based Verification/ Type Approval	Shipboard Spot-Checks
BWE	Physical dilution		
	Biological dilution		Yes
	Presence biological indicators		Yes
BWT	Percent/log reduction in live organisms	Yes	Yes
	No detectable live organisms above a size limit	Yes	Yes
	Limited density of live organisms (above a size limit or within taxa)	Yes	Yes

4. HYBRID METHODS FOR COUNTING, SORTING AND SIZING

4.1. Description

Hybrid methods involve the combination of two analytical technologies e.g., fluorescence detection technology combined with imaging technology, to deliver a more automatic and comprehensive approach to particle analysis. Only one method hybridizing two technologies is currently available:

- ***4.1.1. Imaging Flow Cytometry (e.g., FlowCAM by Fluid Imaging Technologies)***

This hybrid method uses an imaging flow cytometer to count, image and analyze individual particles in a fluid sample as it passes through the instrument. An image of each particle is saved along with conventional flow cytometry data in Microsoft-compatible programs. This includes

but is not limited to chlorophyll *a* and phycoerythrin fluorescence and light scatter. The time of collection and dimensions (length, width, size, area) of the particle are also recorded and stored. Each particle is kept in focus by FlowCAM's patented imaging system. Three visualization modes, with four levels of magnifications, 20X, 10X, 4X and 1X, also allow users to target specific cell types or particles. The system is capable of analyzing particles ranging 1 µm to 3 mm (e.g phytoplankton and zooplankton). Both benchtop (fig. 8) and submersible systems are available. The benchtop system is ideal for analysis of discrete samples or for continuous monitoring of closed systems. An optional pre-installed web server can also provide remote access. Sample processing times are adjustable and range from 1 mL/minute to 12 mL/minute. The system's user interface provides real-time viewing of analysis and particle images in both capture and live video modes. The system can also be integrated with other instruments, such as bulk fluorometers or temperature and salinity monitors. Visit www.fluidimaging.com for more information.

Fig. 8. FlowCAM by Fluid Imaging Technologies

4.2. Functional and Operational Fit (Present)

- ***4.2.1. Functional***

Hybrid methods can achieve a wide range of functions relative to ballast water analysis, including:

- ✓ Count and size organisms automatically.
- ✓ Provide still visual images of organisms for taxonomic identification by an expert.
- ✓ Transmit real-time information offsite.

However, several limitations constrain the usefulness of this type of technology for ballast analysis. For example, hybrid methods cannot:

- ✕ Differentiate live/dead organisms, except where a reliable live/dead stain is available.
- ✕ Analyze large volumes of samples quickly.
- ✕ Provide opportunity to manipulate the subject organisms if more visual information is needed for sizing or identification.

Table 8 summarizes the functional capabilities of hybrid methods for each major taxonomic group.

Table 8. Functional capabilities of hybrid methods by major taxonomic group

	Zooplankton	Phytoplankton	Bacteria
Taxonomic identification	Yes	Yes	
Organism condition	Potential	Potential	
Organism size	Yes	Yes	
Organism enumeration	Yes	Yes	
Bulk measurements of viable biomass		Potential	

- ***4.2.2. Operational***

Sample preparation requires moderate skill, and greater expertise is required for data analysis. These devices yield a tremendous amount of information, some of it useful and much of it not. Yet researchers will have to sift through all of it. Analysis therefore will also be extremely time-consuming and labor intensive. Available systems can process only a limited flow-rate. Higher throughput systems would make them more useful for ballast discharge analysis. Submersible systems have been developed and may be of use to ballast water applications. Bench top systems may also be used onboard ships if properly secured, but the systems are somewhat expensive.

- ***4.2.3. Conclusion***

In terms of specific BWE/BWT analyses, hybrid methods could aid:

- ✓ Shipboard spot-check evaluations of BWE standards (analyzing for indicator near-coastal or high seas organisms).
- ✓ Shore-based verification or approval of BWT systems of spiked organisms for which metabolic dyes are available.
- ✓ Spot-check evaluations of BWT physical separation devices (providing more information than particle counters on the dimensions and nature of particles/organisms passing through the treatment system).

4.3. Prospects and Timeliness for Improved Relevancy

Hybrid methods currently provide both bench-top and in-situ real-time data on numbers, composition and size of zooplankton and phytoplankton, as well as bulk counts of chlorophyll *a* and phycoerythrin fluorescence. The added ability of these systems to take storable and sortable digital images of individual organisms is of benefit to researchers in terms of building taxonomic libraries of source ports, and coastal versus oceanic species compositions. Use of live/dead stains

could enhance the utility of these methods, and allow them to substitute for more conventional methods.

Table 9 summarizes the current and potential regulatory applications of hybrid methods for each type of BWE and BWT discharge standard.

Table 9. Current and potential regulatory applications of hybrid methods for each type of BWE and BWT discharge standard

	Type of Standard	Shore-Based Verification/ Type Approval	Shipboard Spot-Checks
BWE	Physical dilution		
	Biological dilution		Yes
	Presence biological indicators		Yes
BWT	Percent/log reduction in live organisms	Yes	Potential
	No detectable live organisms above a size limit	Yes	Potential
	Limited density of live organisms (above a size limit or within taxa)	Yes	Yes

5. MOLECULAR DETECTION METHODS

5.1. Description

Molecular detection methods use nucleic acid signatures of organisms to detect taxonomy and/or viability. Over time, these technologies have become better candidates for use on ballast treatment applications because more genomes of taxa entrained in ballast water are being mapped, and the units themselves are being packaged with broader capabilities. While microarrays, microfluidics, and nanotechnologies may also one day be relevant to ballast treatment applications, at this time, they require further refinement and development. Four fundamental approaches to molecular detection are currently available:

- ***5.1.1. Polymerase Chain Reaction (PCR)***

This molecular detection method is a common approach used to create copies of specific fragments of DNA via primer extension of nucleic acid (fig. 9). A primer is a short segment of nucleotides complementary to the section of DNA being amplified. Primers are annealed to the denatured DNA template to provide an initiation site for the elongation of the new DNA molecule. Primers can either be specific to a particular DNA nucleotide sequence or they can be universal. PCR is currently the most sensitive technique available to accurately and rapidly amplify and detect low abundance mRNA and minute amounts of DNA. Because DNA is an

extremely stable genetic material, PCR methods can detect the presence of target species or particular strains of species in samples, even after cell death and/or deterioration. The essential criteria for any DNA sample are that it contain at least one intact DNA strand encompassing the region to be amplified. The PCR process involves preparation of the sample, the master mix and the primers, followed by detection and analysis of the reaction products. The product of the PCR is a fragment or fragments of DNA of defined length. A sample of the reaction product can then be loaded, along with appropriate molecular-weight markers, onto an agarose gel and visualized under UV trans-illumination to confirm that the fragments belong to the target species of interest by comparing product bands. PCR is especially useful for searching out target organisms that are difficult or impossible to culture, such as many kinds of bacteria and viruses. The entire process takes 2-3 hours, with results of the analysis available using Microsoft-compatible programs. For more information visit www.appliedbiosystems.com.

Fig. 9. GeneAmp PCR system by Applied Biosystems

- ***5.1.2. Quantitative PCR***

In contrast to PCR, Quantitative PCR is a molecular detection technique used to determine the concentration of organisms present in a sample (fig. 10). The process involves the measurement of PCR amplification as it occurs, cycle-by-cycle, with quantitative measurements made in the highly reproducible exponential phase of PCR. Compared to traditional techniques, the process enables extremely accurate and precise quantification over a large dynamic range, providing for estimates of the amount of target DNA or organisms that were originally present in the sample. The approach has been demonstrated to be useful for quantitative analysis of microorganisms in environmental samples. The entire process takes 2-3 hours, with results of the analysis available using Microsoft-compatible programs. Visit www.appliedbiosystems.com for more information.

Fig. 10. Real-Time PCR system by Applied Biosystems

- ***5.1.3. Laser Scanning Cytometry (e.g. Scan RDI by Chemunex)***

This molecular detection approach employs a laser scanning cytometer to detect and enumerate the presence and number of harmful bacteria and protozoans including *E. coli,* coliforms, *Cryptosporidium,* and *Giardia*. The technology (fig. 11) was developed as a rapid and sensitive device for the analysis of highly diluted fluorescence-labeled cells in water samples without the need for cell growth. The technology involves three steps: (1) membrane filtration of the sample; (2) cell labeling using direct fluorescent labeling of individual, metabolically active cells based on proven reagent technology; and (3) laser scanning that allows all microorganisms present to be detected and counted within 3 minutes. The technology is characterized by its ability to detect and enumerate 1 to 5,000 targeted cells spread over a membrane without dilution. The automated system delivers direct viable cell counts within 90 minutes. In addition, a scan map display shows the precise location of each individually detected microorganism. An optional microscope attachment can provide fast visual result confirmation. Visit www.chemunex.com for more information.

Fig. 11. Scan RDI by Chemunex

- ***5.1.4. Matrix Assisted Laser Detection Ionization (MALDI) Mass Spectrometry***

This molecular detection approach is a form of mass spectrometry that determines mass-to-charge ratio based on travel time of molecules through the analyzer (fig. 12). The technique provides species-specific spectra, and is useful for identifying indicator species. The process involves pulsing of a laser beam onto a laser-absorbing matrix material co-crystallized with analyte molecules onto the sample support surface before insertion into the vacuum system of the mass spectrometer. MALDI analysis consists of two steps: sample preparation and mass spectral analysis. Samples are typically prepared in the concentration ratio of 1:104 analyte to matrix in a suitable solvent such as water, acetone, or tetrahydrofuran. A few microliters of this mixture is deposited onto a substrate and dried, and the solid mixture is then placed into the mass spectrometer. Statistically-based algorithms are used to discern “fingerprints” of target species and to discern individual species from mixtures. The technique is fast, requires minimal liquids/consumables, and is femtomole sensitive. Visit www.serdp.org/research/CP/CP-1248.pdf for more information.

Fig. 12. MALDI MS by Applied Biosystems

5.2. Functional and Operational Fit (Present)

- ***5.2.1. Functional***

Molecular detection methods can achieve a wide range of functions relative to ballast water analysis, including:

- ✓ Detect the presence or absence of indicator organisms and species difficult to culture.
- ✓ Quantify the presence of target or specific species in samples.

Limitations which constrain the usefulness of this type of detection technology for ballast analysis include that such devices cannot:

- ✕ Distinguish organism viability.
- ✕ Detect or quantify all species in a sample; only target species.
- ✕ Provide bulk measurements of biomass.
- ✕ Quickly assess taxonomic composition and sizes of all organisms within a concentrated sample.

Table 10 summarizes the functional capabilities of molecular detection methods for each major taxonomic group.

Table 10. Functional capabilities of molecular detection methods by major taxonomic group

	Zooplankton	Phytoplankton	Bacteria
Taxonomic identification	Yes	Yes	Yes
Organism condition			
Organism size			
Organism enumeration	Potential	Potential	Potential
Bulk measurements of viable biomass			

- ***5.2.2. Operational***

Molecular detection methods require highly trained technicians to both prepare the sample and analyze the results. The process is also extremely labor intensive. It is unlikely that these methods could be a standard procedure performed by members of a ships' crew in the near future. Samples are transportable, extremely stable, and available for long-term storage. Only a minute concentration is required for analysis. Systems are too delicate and bulky for use onboard ships. Though methods range, most are somewhat expensive. Further development and refinement of techniques, particularly PCR-based methods, however, may see a reduction in cost and increase in portability over time.

- ***5.2.3. Conclusion***

Molecular detection methods are currently useful for:

- ✓ Shipboard BWE evaluations to assure that specific harmful organisms are not present in ballast water (such as red tide or *Vibrio sp*.).
- ✓ Shipboard or land-based BWT evaluations of physical separation systems, provided probes are available for indicator organisms**.**

5.3. Prospects and Timeliness for Improved Relevancy

Process miniaturization and automation could increase applicability to the shipboard environment. The methods could be used to:

- ✓ Conduct shipboard spot-checks of BWE, if probes are developed for more indicator organisms of ecological significance.
- ✓ Conduct spot-checks against a BWT standard with respect to all taxa if a method of live-dead differentiation is developed.

Table 11 summarizes the current and potential regulatory applications of molecular detection methods for each type of BWE and BWT discharge standard.

Table 11. Current and potential regulatory applications of molecular detection methods for each type of BWE and BWT discharge standard

	Type of Standard	Shore-Based Verification/ Type Approval	Shipboard Spot-Checks
BWE	Physical dilution		
	Biological dilution		
	Presence biological indicators		Yes
BWT	Percent/log reduction in live organisms		Potential
	No detectable live organisms above a size limit		Yes
	Limited density of live organisms (above a size limit or within taxa)	Potential	Potential

6. BIOCHEMICAL VIABILITY ASSAYS

6.1. Description

The theory behind biochemical viability assays is that organisms have signature biochemical profiles when metabolically active or inactive (dead). Four fundamental approaches to biochemical viability assays are available:

- ***6.1.1. Electron Transport System (ETS) Assay***

This biochemical viability approach uses an enzyme assay to determine cell viability by measuring activity of the ETS. The ETS is a series of biochemical steps by which energy is transferred from a higher to lower level. Electron transport is vital in both photosynthesis and aerobic respiration. The ETS assay is a method of determining the potential oxygen consumption of an organism by measuring the enzymatic activity of the rate-limiting step in oxygen use of adenosine tri-phosphate assay production. The process generally involves sample dilution, extraction, filtration or centrifugation, and spectrophotometric analysis. Larger sample volumes can be filtered. The entire test is usually completed within several minutes.

- ***6.1.2. Adenosine Tri-Phosphate (ATP) Assay***

This biochemical viability approach uses an enzyme assay to determine total viable biomass as well as cell physiological condition in samples. All living organisms contain and use ATP as their main energy source. When cells die, most of their ATP is lost. The amount of ATP in a living cell is proportional to its volume. ATP assays are a rapid, sensitive, inexpensive, and simple method for measuring total viable biomass and involve only two steps. First, the ATP is extracted from the microorganisms, most commonly using detergents, solvents or acids which dissolve parts of the cell wall, allowing the ATP to escape to the water surrounding the cell.

Second, an enzyme is added to the ATP solution and the light produced is measured using spectrophotometry and compared to a known standard. This comparison permits the conversion of instrument readings from the sample to ATP concentration. Then, from ATP concentration the amount of biomass is indicated. The entire test is usually completed within several minutes. The measurement of ATP solutions typically only requires 5 to 15 seconds. ATP extraction times vary depending on the method and the type and condition of the organisms. In many applications, extractions can be adequately completed in less than a minute. ATP biomass tests can be performed manually or they can be automated to varying degrees.

- ***6.1.3. Chlorophyll a Extraction***

This biochemical viability approach involves the extraction and analysis of chlorophyll *a* from phytoplankton samples. Chlorophyll *a* is a key biochemical component of photosynthesis. This method provides an estimate of viable phytoplankton biomass in a given sample, but must be coupled with information on phytoplankton species composition to derive estimates of organism concentrations. Chlorophyll *a* pigments are extracted from the phytoplankton concentrate with aqueous acetone and the optical density, or absorbance, is measured with a spectrophotometer. Whole water or filtered samples can be used to form the concentrate. Samples must be stored away from light and in temperatures below freezing to prevent further chlorophyll *a* degradation prior to analysis. Time for analysis varies with method used and can take up to 24 hours.

- ***6.1.4. Phytoplankton Stress/Death Enzyme***

This biochemical viability approach involves the use of DNA-specific stains in combination with flow cytometry to determine viability of phytoplankton cells. The stains are reliable and easy to use. For example, SYTOX Green will only stain the cellular DNA of cells whose membranes have been compromised, i.e., those with reduced viability. Healthy cells are not stained by the dye, allowing a clear separation of live/dead cells. The stains are generally added to the suspended sample and left to incubate before being put through a flow cytometer. The type of stain being used determines the time of incubation. Typically it is in the range of minutes to hours. Visit www.probes.com for more information.

6.2. Functional and Operational Fit (Present)

- ***6.2.1. Functional***

Biochemical viability assays provide one function relative to ballast water analysis:

- ✓ Bulk indicators of total live biomass of the target taxonomic group.

Limitations that currently constrain the usefulness of this type of assay for ballast analysis are that this tool currently does not:

- ✕ Discriminate between species and size classes.
- ✕ Differentiate biochemical markers recently released from a dead organism, and that released from a live organism in a sample.

Table 12 summarizes the functional capabilities of biochemical viability assays for each major taxonomic group.

Table 12. Functional capabilities of biochemical viability assays by major taxonomic group

	Zooplankton	Phytoplankton	Bacteria
Taxonomic identification			
Organism condition			
Organism size			
Organism enumeration			
Bulk measurements of viable biomass	Yes	Yes	Yes

- ***6.2.2. Operational***

Sample preparation though relatively simple, is an extremely sensitive process. Chlorophyll *a* samples must be stored correctly in dark, cold areas prior to analysis to avoid deterioration. ATP and ETS analysis must occur immediately after collection to avoid death or deterioration of live organisms. Although viability assays require skilled technicians, the processes are relatively easy to carry out. Results are also generally available within a short period of time. Some methods do require bulky equipment, and for that reason are less appropriate for onboard analysis. However, methods are moderately priced.

- ***6.2.3. Conclusion***

Biochemical viability assays can be used in:

- ✓ Land-based BWT verification and approval to evaluate for treatment effects on pure cultures of microorganisms and phytoplankton using a percent reduction standard relative to intake or control (they yield little or no information on absolute numbers of organisms).
- ✓ Land-based BWT verification of presence/absence of live organisms in pure culture above a certain size threshold.

6.3. Prospects and Timeliness for Improved Relevancy

Biochemical viability assays could be used for:

- ✓ Shipboard BWE verification of a biological standard (e.g. using Chlorophyll *a* extraction).
- ✓ BWT spot-checks against a zero concentration (including size-based) or percent reduction standard if methods could be developed and/or ground-truthed for plankton.

Table 13 summarizes the current and potential regulatory applications of biochemical viability assays for each type of BWE and BWT discharge standard.

Table 13. Current and potential regulatory applications of biochemical viability assays for each type of BWE and BWT discharge standard

	Type of Standard	Shore-Based Verification/ Type Approval	Shipboard Spot-Checks
BWE	Physical dilution		
	Biological dilution		Potential
	Presence biological indicators		
BWT	Percent/log reduction in live organisms	Yes	Potential
	No detectable live organisms above a size limit	Yes	Potential
	Limited density of live organisms (above a size limit or within taxa)		

7. BIOCHEMICAL/PHYSICAL MATRIX ASSAYS

7.1. Description

Biochemical/physical matrix assays examine the characteristics of the aquatic matrix to determine its origin or the extent to which a physical operation (i.e. flushing) has been carried out. Two fundamental approaches to biochemical/physical matrix assays are available:

- ***7.1.1. Physical Dye Assays***

This biochemical/physical matrix assay involves the addition of dyes (e.g., methylene blue and Rhodamine WT) to the ballast water. The assay can be used to measure flow patterns, as well as the mixing processes of two different water bodies in relation to both physical mixing and biological mixing (by staining species of plankton). The assay is based on the theory that the rate of dilution of the dye is a direct measure of the rate of dilution of the two bodies of water. Dyes considered for use in ballast water studies must be non-toxic to organisms, environmentally sound, stable over the duration of the study, not prone to bleaching or reactions with chemicals contained in the ballast water and/or sediments, and measurable, even in low concentrations. The dyes can be added to the ballast tank once they are full, or mixed with a small volume of water in the bottom of the tank just before filling. Samples are analyzed in the laboratory for absorbance or fluorescence levels. The type of dye being used and the time for the two bodies of water to mix determines the rate of sample analysis.

- ***7.1.2. Multivariant Chemical/Physical Assays***

This biochemical/physical matrix assay involves the measurement of specific chemical/physical signatures including dissolved organic matter, nitrogen, phosphorus, silicate, salinity, and temperature in the ballast water. The assay is based on the theory that there is a clear difference between the chemical and physical characteristics of ballast water that originated in a coastal area than that originating in the mid-ocean. Signatures considered for use in ballast water studies must be stable and measurable, even in low concentrations. Samples are analyzed either onboard (e.g., salinity and temperature) or in the laboratory (e.g., dissolved organic matter), with the time taken for analysis dependent on the signature being measured.

6.2. Functional and Operational Fit (Present)

- ***7.2.1. Functional***

Biochemical/physical matrix assays provide one function relative to ballast water analysis:

✓ Differentiate ballast water originating in coastal areas from that originating in mid-ocean based on biochemical and physical signatures of sea water.

Limitations that currently constrain the usefulness of these types of assays for ballast analysis are that the tools currently do not:

× Provide any taxonomic information other than to indicate the presence of coastal zooplankton species (e.g., coastal species are stained when a dye is added to the ballast water before the ballast water exchange occurs).

Table 14 summarizes the functional capabilities of biochemical viability assays for each major taxonomic group.

Table 14. Functional capabilities of biochemical/physical matrix assays by major taxonomic group

	Zooplankton	Phytoplankton	Bacteria
Taxonomic identification	Potential		
Organism condition			
Organism size			
Organism enumeration			
Bulk measurements of viable biomass			

- ***7.2.2. Operational***

Sample preparation especially for physical dye assays is an extremely difficult task. Researchers need to ensure complete mixing of the dye with the ballast water prior to the addition of a second body of water. Dye samples also need to be analyzed in the laboratory, so must be kept stable in the time prior to analysis to avoid deterioration. Laboratory analysis also requires trained professionals, though results are generally available within a short period of time. Some methods do require bulky equipment, and for that reason are less appropriate for onboard analysis. However, methods are moderately priced.

- ***7.2.3. Conclusion***

Biochemical/physical matrix assays can be used in:

✓ Shipboard BWE verification and spot-checks of a physical dilution standard.

7.3. Prospects and Timeliness for Improved Relevancy

Biochemical/physical matrix assays could be used for:

✓ Shipboard BWE verification and spot-checks of a biological-based standard (dye studies only).

Table 15 summarizes the current and potential regulatory applications of biochemical viability assays for each type of BWE and BWT discharge standard.

Table 15. Current and potential regulatory applications of biochemical/physical matrix assays for each type of BWE and BWT discharge standard

	Type of Standard	Shore-Based Verification/ Type Approval	Shipboard Spot-Checks
BWE	Physical dilution	Yes	Yes
	Biological dilution	Potential	Potential
	Presence biological indicators	Potential	Potential
BWT	Percent/log reduction in live organisms		
	No detectable live organisms above a size limit		
	Limited density of live organisms (above a size limit or within taxa)		

REVIEW OUTCOME BY STANDARD TYPE

The output of the presentation and review process is presented below by BWE and BWT standard type. The review summarizes whether analytical tools are relevant now, are likely to be relevant in the near future, or could be developed with sufficient attention. The review also recommends research objectives to increase the potential of the tools to be used in ballast discharge evaluations.

1. BALLAST WATER EXCHANGE APPLICATIONS

As noted, there are three possible types of BWE regulatory standards. Of these, the two most accepted are physical dilution of ballast water with mid-oceanic water and biological dilution of biota with mid-oceanic biota. Summarized below are the observations of Workshop participants relative to the readiness of analytical tools to support these two standard types.

1.1. Prescribed Physical Dilution of Ballast Water with Mid-Oceanic Water

- ***1.1.1. Analytical Tools Available Now***

Relevant analytical tools currently available to verify compliance with a physical dilution standard include:

- ✓ Physical dye and fluorescent sphere studies (aided by computational models of ballast uptake and tank flow dynamics and mixing characteristics).
- ✓ Multivariant chemical/physical assays.

Physical dye and other tracer studies are well suited for BWE type approval, especially when aided by computational models, but limited in their spot-checking application. They require the addition of a known quantity of dye or beads prior to the exchange (e.g. by officials in the source port), followed by sampling after BWE but before discharge in the recipient port. This tool is also restricted in that the assay must be evenly mixed throughout the tank prior to the exchange. Random tank sampling prior to discharge could be a more practical verification mechanism if combined with seawater chemistry probe information to determine compliance.

Recommendations to increase the readiness of currently available analytical tools include:

- ✓ Applied research involving tank mixing trials using stratified sampling before and after BWE of different tank configurations and ship types.
- ✓ Development of models on ballast uptake and tank flow dynamics.

- ***1.1.2. Analytical Tools Likely to be Available in the Near Future/Could be Developed with Sufficient Attention***

Relevant analytical tools likely to be applicable in the near future to assist BWE compliance verification for a physical dilution standard include:

- ✓ Particle sizing systems and counters.
- ✓ Multivariant chemical/physical assays including optical fluorescence and spectrofluorescence of dissolved organic matter.
- ✓ Fluorescent sphere dilution studies.
- ✓ Models of ballast uptake and tank flow dynamics and mixing characteristics.
- ✓ Black box in-line monitoring systems to detect dyes/beads/chemical/physical signatures of discharge

In this situation, black box technologies (e.g. in-line monitoring of ballast uptake and discharge) could be analyzed by regulatory agencies, or the data sent to shore automatically via the Internet before the ship's arrival in the recipient port. The technology could also be combined with decision support systems, (e.g. the Shipping Explorer risk monitoring model currently being developed by Cawthron Institute, New Zealand) and mid-ocean areas that have been designated as safe for carrying out mid-ocean exchanges.

1.2. Prescribed Biological Dilution of Ballast Water with Mid-Oceanic Biota

- ***1.2.1. Analytical Tools Available Now***

Relevant analytical tools currently available to verify compliance with a BWE biological dilution standard include:

- ✓ Visual methods for organism counting, sorting and sizing.
- ✓ Hybrid methods for counting, sorting and sizing.
- ✓ Molecular detection methods.
- ✓ State-of-the-art statistical modeling techniques for data analysis.

Light and scanning electron microscopy and bacteria plate counts have potential application in verifying BWE compliance, as well as for ground-truthing the effectiveness of hybrid methods for counting, sorting and sizing e.g., FlowCAM. This information could then be used to establish port, regional or national scale FlowCAM Clearinghouses whereby ballast water taxonomic experts view electronically transmitted images and make decisions on whether or not the ballast water originated from the mid-ocean. The technology could also be combined with molecular detection methods for selected groups of target organisms (e.g. strictly coastal, mid-ocean and particularly high risk species such as *Vibrio cholera*). Verification systems could employ voyage pathway information to verify compliance (e.g. ballast on relatively long trans-equatorial routes can undergo marked variations in temperatures resulting in mass mortality of various ballast water organisms, unlike shorter routes at similar latitudes for which survivorship may be much higher).

Recommendations to increase the readiness of currently available analytical tools include:

- ✓ Research targeted at determining the beneficial uses hybrid methods, and molecular detection methods in BWE compliance and verification situations.
- ✓ Research on standardized ballast sampling methods to ensure that samples are representative of the tank environment including all biota.

▪ *1.2.2. Analytical Tools Likely to be Available in the Near Future*

Analytical tools likely to be relevant in the near future to assist BWE compliance verification for a biological dilution standard include:

- ✓ Hybrid methods.
- ✓ Fluorescence detection for organism counting and/or sorting.
- ✓ Microscopy combined with optical imagery.
- ✓ Molecular detection methods (e.g., PCR and RNA analysis).
- ✓ Biochemical viability assays (e.g., Chlorophyll *a* extraction).

▪ *1.2.3. Analytical Tools that Could be Developed with Sufficient Attention*

There are numerous analytical tools that upon development could potentially be used in BWE compliance and verification. These include:

- ✓ High-tech Expert Systems aimed at giving regulators a greater understanding of the risks and for selecting appropriate ballast water management options (e.g. a more advance version of Shipping Explorer).
- ✓ Advanced molecular labeling techniques and probes.
- ✓ Plankton biogeography and assemblage typing

2. BALLAST WATER TREATMENT APPLICATIONS

As stated, there is debate over the best approach to expressing a BWT standard. The two fundamental approaches under discussion are use of a process efficiency standard (e.g., log reduction or percent reduction in live organisms relative to control or intake concentrations), and use of zero or absolute limits on discharge concentrations of live organisms, including above a certain size class or within taxa. Summarized below are the observations of Workshop participants relative to the readiness of analytical tools to support these approaches to a standard.

2.1. Percent or Log Reductions in Various Taxonomic Groups Relative to Intake

▪ *2.1.1. Analytical Tools Relevant Now*

Analytical tools currently available to verify compliance with a BWT percent or log reduction standard include:

- ✓ Visual methods for counting and sorting.
- ✓ Biochemical viability assays.
- ✓ Fluorescence detection methods.
- ✓ Hybrid methods.

Visual methods for counting and sorting remain the best analytical tools currently available to verify compliance with a standard expressed as percent reduction relative to intake for zooplankton. In this situation, a concentrated sample (possibly facilitated by live/dead stains) can be analyzed using either conventional microscopy or a microscope attached with a camera to provide precise information on numbers of live organisms in discharge relative to an intake density. The addition of a camera to the microscope may be used by regulators to collate an imaging library, identify indicator species, or species of concern, and help with remote verification and compliance, whereby ships could send still pictures or short film clips of the ballast water sample to the approaching port prior to entry approval.

In terms of phytoplankton analysis, changes in bulk concentrations of phytoplankton (algal biomass) due to treatment can be measured using a biochemical viability assay such as Chlorophyll *a* extraction or fluorescence detection methods such as flow cytometers. Flow cytometers can also be used to provide immediate bulk and count data on Chlorophyll *a* relative to intake. The FlowCAM system and/or image analysis may enhance taxonomic qualifications. A variation of this approach would be to conduct bulk assessment of Chlorophyll *a* following a grow-out period to determine grow-out potential. Care is required to select the best media for culturing the phytoplankton.

Conventional methods such as bacteria plate counts of colony forming units can be used to test for microorganisms in samples to compare log reductions between intake and discharge samples. Samples can be filtered, plated, and grown onboard the ship. Care is required to maintain a sterile environment, constant ambient temperature, and appropriate media for culturing.

Recommendations to increase the readiness of currently available analytical tools include:

- ✓ Research using conservative size bins to estimate taxonomic reductions so that biochemical viability assays such as ATP and ETS evaluations can be undertaken to compare discharge and intake samples.
- ✓ Retrofitting in-line sample ports to ballast intake and discharge lines to aid with sample collection.

▪ *2.1.2. Analytical Tools Likely to be Relevant in the Near Future/Could be Developed with Sufficient Attention*

Analytical tools likely to be relevant in the near future to verify compliance with a percent or log reduction standard include:

- ✓ Fluorescence detection methods.
- ✓ Hybrid methods for counting, sorting and sizing.

Recommendations to increase the readiness of analytical tools likely to be relevant in the near future include:

- ✓ Development of live/dead stains.
- ✓ Mechanization of the entire sampling process to reduce the need for personnel involvement and scientific supervision.

The comparison of intake and discharge samples to determine percent or log reduction would be greatly facilitated by development of live/dead stains. In this situation, and if the presence of a whole zooplankton indicates life, live/dead assessments could be simplified to a count using image analyzers and fluorescence detection methods.

2.2. No Detectable Live Organisms above a Size Limit

▪ *2.2.1. Analytical Tools Relevant Now*

Analytical tools currently available to verify compliance with a no detectable live organism discharge concentration standard include:

- ✓ Particle counting and sizing systems.
- ✓ Fluorescence detection for organism counting and/or sorting.
- ✓ Visual methods for counting, sorting and sizing.
- ✓ Biochemical viability assays.

Particle counting and sizing systems could be used to verify compliance with a zero discharge standard of live organisms above a certain size if the technology is a physical separation device or if samples are collected using a device that separates organisms by size. They are not helpful if the technology also involves a biocidal treatment step such as UV irradiation. In terms of taxonomic applicability, visual microscopy of a concentrated sample (possibly facilitated by live/dead stains) will provide precise information on the numbers of live zooplankton per liter contained in the discharge. There are no analytical methods currently available to distinguish live phytoplankton from dead given in unknown assemblages. Grow out methods combined with chlorophyll *a* extraction could be used to determine growth potential but only if a wide range of growth media are employed because the organisms in the discharge will have unknown growth requirements.

Recommendations to increase the readiness of currently available analytical tools include:

- ✓ Taps in the ballast discharge lines and research as to how representative particle distributions generated by particle counting and sizing systems are to the actual discharge stream.
- ✓ Development of on-line (in situ) counters that can transmit real-time information over the Internet.
- ✓ Development of portable equipment.

- ***2.2.2. Analytical Tools Likely to be Relevant in the Near Future***

Analytical tools likely to be relevant in the near future to verify compliance with a zero discharge concentration standard include:

- ✓ Biochemical viability assays (e.g., ATP and ETS).
- ✓ Hybrid methods for counting, sorting and sizing.
- ✓ Particle counting and sizing systems.

ATP and ETS assays could be a useful tool to determine whether there are live organisms in the discharge. However these approaches are not useful if the standard includes a certain number of allowable organisms because they do not distinguish types of organisms nor quantity of organisms. Unfortunately, there is no good way to determine number of viable phytoplankton unless it is a well studied indicator species that can be stained.

Recommendations to increase the readiness of analytical tools likely to be relevant in the near future include:

- ✓ Development of a flow cytometer to accommodate larger sample and species sizes could be of use when evaluating ballast water for the presence of live organisms in discharge samples.
- ✓ Reduction in the minimum particle size limit for systems like the FlowCAM to accommodate microorganisms.
- ✓ Research on side scatter specific to particle counting and sizing systems to provide information on shape, possibly allowing investigators to distinguish taxonomic groups and life stages.

- ***2.2.3. Analytical Tools that Could be Developed with Sufficient Attention***

Attachment of a "black box" type recorder to the discharge line (or an offshoot of the discharge line that deviates only a small but representative sample of the water being discharged through the recorder) and provides a print out of the number of live organisms in the discharge over the entire discharge duration, could be another possible BWT verification tool. The system could be based on the idea of a litmus test whereby when a live organism hits the test paper its presence is marked on the print-out. The system could also be used in conjunction with a filter mechanism that allows only species above a certain size class to make imprints on the test strip. Development of such a system would overcome obstacles associated with sample collection, concentration and analysis as well as the need for technical support, as no water sample would actually be collected.

2.3. Limited Density of Live Organisms above a Size Limit or Within Taxa

- ***2.3.1. Analytical Tools Relevant Now***

Analytical tools currently available to verify compliance with a limit on discharge concentration standard include:

- ✓ Fluorescence detection methods (e.g., flow cytometers).
- ✓ Hybrid methods for counting, sorting and sizing (e.g., FlowCAM and optical zooplankton counters).
- ✓ Visual methods of counting, sorting and sizing.

Although somewhat limited in application to test for zooplankton, flow cytometers could be used to measure absolute concentrations of mixed assemblages of bacteria and phytoplankton, as well as to distinguish bacteria (stained) and phytoplankton within the sample. In terms of zooplankton, hybrid and visual methods such as FlowCAM, image analysis and optical zooplankton counters could provide absolute concentration determinations, as well as for phytoplankton. Visual microscopy of a concentrated sample facilitated by live/dead stains will also provide precise information on the numbers of live zooplankton per liter contained in the discharge.

- ***2.3.2. Analytical Tools Likely to be Relevant in the Near Future***

Analytical tools likely to be relevant in the near future to verify compliance with a limit on discharge concentration standard include:

- ✓ Biochemical viability assays (e.g., ATP and ETS).
- ✓ Fluorescence detection methods.
- ✓ Particle counting and sizing methods.
- ✓ Hybrid methods for counting, sorting and sizing.

Analysis of size fractions using ATP and ETS could be a useful tool to determine whether there are live organisms in the discharge above a certain size limit. These approaches are not useful if the standard includes a certain number of allowable organisms above a certain size because they do not distinguish types of organisms nor quantity of organisms. Size fractionation, however, could be used in combination with ETS assays to provide more analytical measurements.

Fluorescence detection methods may also have some application in the future to evaluate BWT performance relative to specific indicator or spiked organisms. Methods could be used in combination with another technology such as image analysis software to determine organism counts by taxa as well as bulk counts.

In addition, particle counting and sizing methods could be linked with qualitative information about a dominant species in a particular size range such that the particle data could suggest species' presence and densities. The ability to make this extrapolation, however, may be limited to organisms that occur in distinctive "blooms", and which are not in the same size category as inanimate particles e.g., dinoflagellates.

Recommendations to increase the readiness of analytical tools likely to be relevant in the near future include:

- ✓ Research on ATP assays applicable to phytoplankton and zooplankton.

Though biochemical viability assays already exist for microorganisms, there will need to be some intensive research on ATP assays applicable to phytoplankton and zooplankton. This technique will have to be developed and calibrated with information on the speed at which ATP degrades after death.

- ***2.3.3. Analytical Tools that Could be Developed with Sufficient Attention***

PCR and other molecular based methods could also be used to detect the presence or absence of species via ballast water kits or protocols. In this case, the target could be specific species, strains of species, or broader groups of taxa. Investigators need to realize, though, that detection of a particular species using a molecular method does not necessarily translate to the detection of viable organisms. DNA is a relatively stable molecule under a variety of environmental conditions that would "kill" organisms. This potential problem can be overcome if a period of growth is a part of the protocol before the molecular method is applied. Therefore, only viable organisms would be detected or enumerated in the modified method.

Additionally, insertion of an optical plankton counter-type instrument into the discharge line or an offshoot of the discharge line that counts, analyzes, and takes images of the plankton being discharged and transmits this information to a central computer for analysis, may also be a possibility. This information can ultimately be summarized using a specific software program and sent via the Internet to the approaching port for evaluation of BWT compliance.

3. SUMMARY OF TOOL AVAILABILITY BY STANDARD TYPE

Participant comments suggest the following answers to the fundamental questions that inspired the Workshop. It is important to note that the availability of tools is dynamic in that new tools could be developed with sufficient attention. An understanding of what is available now, however, informs decisions of how to handle approvals/spot checking in the immediate future, *vis a vi*s the long term, and how to focus methods development efforts over time.

1. Are tools available to support approval of BWT against all types of standard under discussion, given a shore-based test pad arrangement with known intake and discharge quality?

- ✓ Analytical tools are available to approve treatment systems in a shore-based test pad scenario against a process efficiency standard.
- ✓ Tools are available for zooplankton analysis for evaluation against both standard types (indeed, because the intake quality is known in the shore-based test pad context, there is little difference in analytical demands of a discharge standard versus process efficiency standard).
- ✓ Methods development is needed to determine live/dead status of individual phytoplankton and bacteria particles in keeping with a discharge limit standard.

2. Are tools available to support shipboard evaluations against all types of standards for BWT and BWE currently under discussion?

- ✓ Analytical tools exist to support evaluations against a BWT process efficiency standard relative to control, but such comparisons are limited without concomitant ballast uptake characterizations, and this exercise is encumbered by heavy logistical demands to obtain comparable intake and discharge samples.
- ✓ Analysis against a BWT discharge quality standard prescribing no detectable live organisms over a certain size can be achieved with limited methods development.
- ✓ Analysis against a BWT discharge quality standard involving limited live organisms across taxa (like IMO) is not currently feasible, and would require substantial methods development (see below).
- ✓ There is no difference in analytical requirements between BWE biological-based process efficiency and discharge quality standards, and though labor intensive, this analysis could be undertaken now.

3. What is the status of tools to analyze ballast discharge from ships consistent with the discharge limit standard proposed by IMO?

- ✓ For zooplankton analysis, tools exist, though the methods are labor-intensive, not automated, do not provide immediate results; require skilled operators and trained taxonomists to analyze results; and are expensive.
- ✓ For phytoplankton, there are no methods to enumerate live particles per unit volume for an unknown assemblage, and achieving this task will require substantial methods development.
- ✓ For bacteria, methods require a grow-out arrangement, are not automated, and are operator and time intensive. Also, species-specific media and constant temperatures must be used and maintained during the incubation process.

4. How far are we from having them?

- ✓ With directed research and development, more practical tools could become available, but especially tools for live phytoplankton enumeration could take many years to develop and verify.

5. What will be necessary to usher in their development?

- ✓ Ratification of IMO convention.
- ✓ Authorization of federal standards for ballast management.
- ✓ A standardized and representative sampling protocol.
- ✓ Funding to support methods development work

6. What are the characteristics of the ideal discharge evaluation system, and what research objectives did workshop participants recommend that could make the ideal a reality?

- ✓ A uniform sample collection process accompanied by "full-service" PCR probes would be ideal. That is, probes which can detect live organisms across taxonomic groupings, and enumerate them. This method, however, could be decades away, even given a targeted methods development effort. A standard prescribing zero live organisms per unit volume will require less methods development in that enumeration is no longer necessary. This type of standard would also clearly be more protective of the environment, but technologies to deliver this level of treatment that do not have accompanying environmental hazards may take some time to evolve.

CONCLUSION

Clearly, all categories of analytical tools considered by Workshop participants for use in ballast discharge regulatory situations have some applicability. The type of standard that is chosen in both BWE and BWT contexts will however heavily influence the applicability of specific tools to any compliance related functions, and analytical methods are not currently available to support analysis against all standards under discussion. The most difficult analytical challenges appear to lie with determinations of absolute numbers of live phytoplankton in a sample containing an unknown species composition. Questions around the reliability of metabolic stains and biochemical indicators for an unknown assemblage hamper their use. Grow-out experiments also depend on prior knowledge of the optimal conditions/media for the organisms being cultured. Relative comparisons of phytoplankton biomass as represented by Chlorophyll *a* (as per a percent reduction) are currently more feasible.

Over time, ATP analysis could be a tool for determining whether no live organisms exist above a certain size, or within the sample absolutely, but currently protocols exist only for bacteria. Such an approach cannot be used if there is an allowable number of algal particles per liter, as the amount of ATP per organism in an unknown assemblage will be difficult or impossible to assess. The rate at which ATP decreases over time also needs to be taken into account and researched. In all cases, the identification of indicator organisms or taxa would greatly enhance the number of analytical tools available to carry out monitoring.

Regarding verification of BWE, it will be easier to estimate physical dilution using ship logs and dye studies, than biological dilution in the form of a spot-check. Multi-parameter sea probes have been proposed but their use in a regulatory system would require intensive data gathering on the signatures of coastal waters around the world. Otherwise, cross-checking ships' logs of various operations can provide some verification of records. Biological verification could be possible in the future using remote microscopes if near coastal indicator organisms could be agreed. Over time, such an approach could be automated using a FlowCAM or molecular detection methods.

In terms of BWT verification, methods currently available for use in this context include flow cytometers, FlowCAM and optical zooplankton counters, along with conventional microscopy.

Physical separation devices such as particle analyzers may also be of relevance for treatment systems that remove particles and organisms from the ballast water. With development, ATP and ETS assays could be useful tools to determine whether there are live organisms in the discharge. However these approaches are not useful if the standard includes a certain number of allowable organisms because they do not distinguish types of organisms nor quantity of organisms. Fluorescence detection methods may also have some application in the future to evaluate BWT performance relative to specific indicator or spiked organisms. These methods could be used in combination with another technology such as image analysis software to determine organism counts by taxa as well as bulk counts. The development of a flow cytometer to accommodate larger sample and species sizes could also be of use when evaluating ballast water for the presence of live organisms in discharge samples. PCR and other molecular based methods, and automated discharge recorders show promise for future applications but a great deal of research and development is required.

Overall, both BWE and BWT discharge analysis would be greatly facilitated by wide-spread installation within the commercial fleet of similar in-line sample ports in the ballast intake and discharge piping. For basic research, sample ports combined with a uniform type of access to ballast tanks would significantly enhance data quality. All of the analytical tool options described will require a great deal of method and technology development. Tools that are mechanized, reduce investigator supervision and sample preparation, are portable and require little or no scientific background are more ideal. Workshop participants urge that agencies interested in BWT development also assist in development of analytical tools for evaluating biological characteristics of ballast discharge.

In general, Workshop participants recommend:

- ✓ Addition of in-line sampling ports on ships' ballast lines to enable comparable sampling.
- ✓ Modification of existing tank access points (e.g. man-hole hatches) on ships for use in research and analytical tool development.
- ✓ Development or refinement of techniques for concentrating samples that do not alter viability of the live organisms.
- ✓ Development of more live/dead stains or other biochemical viability indicators, such as enzymes.
- ✓ Development of better methods for capturing the full range of organisms in the preserved samples.

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NORTHEAST
MIDWEST
INSTITUTE

218 D St, SE
Washington DC 20003
Ph. 202 544 5200
www.nemw.org