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STRATEGY FOR TESTING THE EFFICACY OF BALLAST WATER TREATMENT TECHNOLOGIES

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N O T I C E

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16. Abstract (MAXIMUM 200 WORDS

One of the principal vectors of aquatic invasive species introductions is the transport of organisms in ballast water. The primary management practice to reduce such introductions during routine ballasting operations is mid-ocean ballast water exchange (BWE). Due to BWE limitations (e.g., safety, route, efficiency), ballast water treatment (BWT) technologies to remove or inactivate entrained organisms are being developed. BWT technology is in the very early stages of development as are the testing approaches to gauge BWT efficacy. During reviews of various BWT systems for the U. S. Coast Guard, it became clear that while the testing methodologies may be technically sophisticated, the test programs generally employ inappropriate measures of system effectiveness and frequently lack adequate experimental design. BWT effectiveness must be based upon either species-specific physical removal or organism viability/propagation.

This report provides an overview of concepts needed for effective testing of BWT technology efficacy. The focus is to relate both theoretical and design issues to BWT test program development and conduct. Examples from experiences with Coast Guard's scientific audits, workshops, and available literature illustrate key principles and methodological problems encountered. Also discussed is the need for research into new methods for ascertaining viability, and investigations to discover surrogate species whose inactivation represents the response of a broad spectrum of taxa identified in ballast water.

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EXECUTIVE SUMMARY

Ballast water has been identified as a major pathway for the introduction of aquatic nuisance species (ANS) in global coastal oceans and major freshwater systems worldwide. The primary management practice to reduce such introductions during routine ballasting operations is mid-ocean ballast water exchange. Due to ballast water exchange limitations (e.g., safety, route, efficiency), ballast water treatment (BWT) technologies to remove or inactivate entrained organisms are being developed. The development of BWT technologies is in the very early stages, as are the testing approaches to gauge treatment performance. It is the immature state of development of the testing approaches, however, that has led to problems quantifying treatment performance. The U.S. Coast Guard found during scientific audits of various BWT systems that it was impossible to determine treatment performance because most of the test programs used inappropriate measures of system effectiveness and frequently lacked adequate experimental design. The audit findings repeatedly demonstrated that the test programs were wrought with enough fundamental problems that their extent and gravity undermined any conclusions and insights that could be drawn about treatment performance of the systems tested.

In order to assess how test programs are generally designed, the testing structure of six BWT test programs were examined. The single most important problem shared among the test programs was the determination of BWT effectiveness based upon changes in biomass or other bulk indicators of population size. Generally, this approach was used in order to simplify the testing program or to accommodate logistical considerations in shipboard testing. Even when conducted at the species level, however, bulk measures almost always underestimate the degree of organism inactivation because they do not measure viability. Failure to measure viability can invalidate significant time and effort spent by technology developers and ship-owners in attempts to quantify BWT capabilities. More importantly, the application of invalid or confounded testing approaches delays the development and implementation of workable BWT technologies.

Because many current technologies rely primarily on creating lethal conditions (e.g., UV, Ozone, biocides) or directly removing organisms (e.g. filtration), "true" BWT effectiveness must be based upon either species-specific physical removal rates or reduction in the numbers of organisms that are

able to reproduce. Because future reproductive capability is exceedingly difficult to quantify for many taxa, viability may be substituted as a conservative measure. The ideal BWT technology test program would be an evaluation of the number and viability of each species before treatment, immediately after treatment, and again after a holding time, as an assessment of moribund members of the population. This would allow for a quantitative determination of treatment effectiveness within and among taxonomic groupings.

This report identifies common methodological problems of the six test programs and presents an overview of concepts needed to properly test BWT technologies. It also discusses the critical need for research into (1) new methods for ascertaining viability over the broad spectrum of taxa covering almost all phyla that have been identified in ballast water, and (2) surrogate species whose inactivation is a proxy for inactivation of a wide spectrum of less robust taxa. The report is not intended to be prescriptive, but to provide general guidelines that will encourage technology developers and researchers to apply appropriate techniques that will result in quantitatively useful data regarding a BWT system's capabilities.

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LIST OF ACRONYMS

ANS	Aquatic Nuisance Species
ATP	Adenosine Tri-phosphate
BWE	Ballast Water Exchange
BWT	Ballast Water Treatment
DNA	Deoxyribonucleic Acid
ETS	Electron Transport System
EEZ	Exclusive Economic Zone
IMO	International Maritime Organization
RNA	Ribonucleic Acid
MPN	Most Probable Number
NISA	National Invasive Species Act
NOAA	National Oceanic and Atmospheric Administration
NRC	National Resource Council
PAM	Pulse Amplitude Modulated Fluorometry
STEP	Shipboard Technology Evaluation Program
USCG	United States Coast Guard
USCG RDC	United States Coast Guard Research and Development Center
USEPA	United States Environmental Protection Agency

1.0 BACKGROUND AND OVERVIEW OF PROBLEM

One consequence of the globalization of trade has been the inadvertent transfer of animal and plant species into geographic regions where normally they are not part of the indigenous population. If conditions exist where proliferation can proceed unchecked by the usual environmental controls (e.g., predation, nutritional status, climate), unwanted changes in the distributions of populations could significantly impact the ecology, economy and human health status of a region (e.g., Wilcove, et al., 1998; Pimentel et al., 2000; Ruiz et. al., 2000a). In coastal marine ecosystems, ballast water is a primary vector by which foreign invaders are introduced. Typically, when cargo is offloaded from ships at a given port, ballast water is simultaneously taken on to maintain trim and stability. This ballast is then transported to the next port of call where it may be discharged for better maneuverability in the shallow waters and again before taking on the next load of cargo.

The volume of ballast water discharged within ports annually can be very large and originate from a wide spectrum of source waters. A single vessel can discharge more than 100,000 tons of ballast water within a small region of a harbor. Annually, more than 79 million metric tons of foreign ballast water derived from one or more foreign sources (e.g., Carlton and Geller, 1993; Smith et al., 1999; Ruiz et al., 2000a; Wonham, 2001) and containing a taxonomically diverse assemblage of nearly all phyla are discharged in U.S. harbors alone (Carlton et al., 1995). This has resulted in colonization by vast numbers of nonindigenous species all over the globe. In North America alone, roughly 400 marine and estuarine nonindigenous species have become established, a significant fraction of these entering through the ballast water pathway (Cohen and Carlton, 1995; Ruiz et al., 1997, 2000b). Some aggressive species expand into open niches unimpeded by natural predators to such an extent that entire ecosystems are altered, often with the displacement of indigenous species that are economically important to the region (e.g., Ruiz et al., 1997; Grosholz, et al., 2000). The U.S. Coast Guard Research and Development Center's (CG RDC) 1999 Research Assessment report (Hiltabrand and Roderick, 1999) discussed the worldwide nature of the problem and concluded that approaches are needed for preventing further introductions of aquatic nuisance species (ANS), rather than the expensive alternative of remediation after an invasion has occurred. For example, in the U.S. nearly $122 billion dollars are spent annually on remediation of all invasive species (terrestrial and aquatic), with more than

$7.3 billion for aquatic invasive species alone. The costs are even greater if economic losses due to bioinvasion-mediated changes in natural populations are considered (Hiltabrand and Roderick, 1999; Harder, 2002).

Since 1990 the U.S. has, through Presidential Executive order and legislation, attempted management of the problem. The National Invasive Species Act (NISA) of 1996 establishes voluntary guidelines for all ships entering the U.S. Exclusive Economic Zone (EEZ), except in the Great Lakes and the Saint Lawrence Seaway where a mandatory program is in place. The major ballast water management approach cited in NISA is exchange of ballast water outside the U.S. EEZ or exchange in other waters where release of organisms does not pose a threat of invasion. NISA also supports the use of ballast water treatment technologies in lieu of BWE as long as they are as effective in removing or inactivating organisms entrained in ballast water. The USCG recently determined, however, that the voluntary program has been ineffective and is currently preparing mandatory regulations for release within the coming year.

The International Maritime Organization (IMO) Resolution A.868(20), "Guidelines for the Control and Management of Ships' Ballast Water to Minimize the Transfer of Harmful Aquatic Organisms and Pathogens," as well as proposed language for a new Annex for the International Convention for the Control and Management of Ships' Ballast Water and Sediments, rely mainly on BWE to minimize the probability of ANS introductions (Dames and Moore, 1999).

BWE is generally viewed to be a short-term approach for limiting introductions of additional invasive species via ballast water. Its attraction is that it can be implemented quickly as it does not require the installation of new shipboard hardware, which in many cases has yet to be developed. BWE allows an immediate, if partial, treatment of a real problem until the science and technology required for treating ballast water becomes available. BWE, however, has limitations. First, for stability and structural reasons, it is not always safe to exchange ballast on the open seas, particularly during rough weather. Second, during coastal transport or within large freshwater systems (e.g., Great Lakes), open-water BWE is not logistically feasible. Third, depending upon tank design, significant numbers of residual coastal organisms are often retained in tanks even after an exchange.

The matter is further complicated in that BWE sets a somewhat undefined standard that is percentage based (e.g., ~95 percent removal) rather than "absolute" (set number of organisms per volume). Since populations of marine organisms entrained in ballast water vary with source water, a percent reduction standard would result in a wide range of population densities in discharge waters being acceptable. The optimal treatment standards would set the absolute numbers of organisms allowable in discharge waters (Waite, 2002), and this is the direction that ballast water management will likely move in the coming years. In many situations, treatment technology will be needed in order to meet any absolute standard.

Awareness of the above issues by the maritime industry has resulted in significant efforts to develop and implement technological alternatives to BWE. These technologies are not limited by sea conditions and have the potential to exceed the effectiveness of BWE in preventing introductions. Although many treatment possibilities are being explored (NRC, 1995; Hallegraeff, 1998; http://www.invasions.si.edu), testing and evaluation is at a very early stage, and no alternative treatments have been successfully demonstrated.

To date, research and development related to bringing new ballast water treatment technologies on-line for shipboard use have been supported primarily through private industry. The industry's technology developers have hired scientists to design test programs for assessing system performance of newly developed treatment technologies. Since there are no standard test approaches or protocols, scientists and engineers are simultaneously developing treatment technologies, along with the necessary test strategies and protocols. While at this early stage, the flexibility in testing approaches has allowed for better site- and treatment-specific test criteria and has encouraged creativity within the scientific community, it has also led to the use of assays that are inappropriate for determining BWT effectiveness.

This problem became evident while the CG RDC was auditing (i.e., evaluating) the performance of several BWT systems that were undergoing tests by technology developers. The intent of the audit program was to provide the CG with a better understanding of the current state of the development of BWT technologies. However, the findings of the audits repeatedly demonstrated that the test programs were wrought with enough fundamental problems that their extent and

gravity undermined any conclusions and insights that could be drawn about the efficacy of any particular treatment system (Roderick, 2004).

The overarching research problem that needs to be addressed by all BWT technology testing is to determine the efficiency of inactivation or removal of all species present in ballast water. The technical issue is how to address this species-specific question, when ballast water has been observed to contain virtually all of the major taxonomic groups that exist in marine systems, plus viruses. The general approach by all test programs reviewed has been to group organisms by general categories: zooplankton (animals), phytoplankton (plants, dinoflagellates), protozoans (ciliates, flagellates, amoeba, etc), bacteria (all), and viruses. However, this does not take into account the wide diversity of organisms that make up each major grouping (Figure 1). It must be noted that the major endpoints of the tree (e.g., Porifera, Mollusca, Diatoms, Red Algae) are typically phyla, each of which normally contain hundreds to thousands of individual species.

Since ballast water comes from all over the world's coastal oceans, treatment must be effective on almost all species of marine life. For example, the zooplankton group includes virtually every group of animals, either as adults or as juveniles. In addition to organisms that live their entire lives as plankton (holoplankton), about 70 percent of bottom dwelling marine invertebrates have planktonic stages (Barnes, 1974), which are assayed as zooplankton in BWT testing. Within the animal or zooplankton group alone, the life history, physical structure and physiology of organisms are highly variable ranging from jelly animals (cnidaria, ctenophores) to shelled mollusks to fish. The complexity for test programs increases further when the size range (less than 1 μm to greater than 1 cm) and biophysiology of the other major groups (plants, bacteria, viruses) are also considered.

In order to assess how test programs are generally designed, we reviewed the testing structure of the four test programs evaluated by the USCG RDC and two other BWT technology test programs for which data were available (Sutherland et al., 2001; Waite et al., 2003; Table 1). The major goal was to determine similarities in experimental design and protocols that were followed, and their appropriateness for assessing BWT efficiency. Special attention was given to the biological portion of the test evaluations to determine if appropriate assays of biological removal/inactivation were being employed, if methodological procedures were being applied

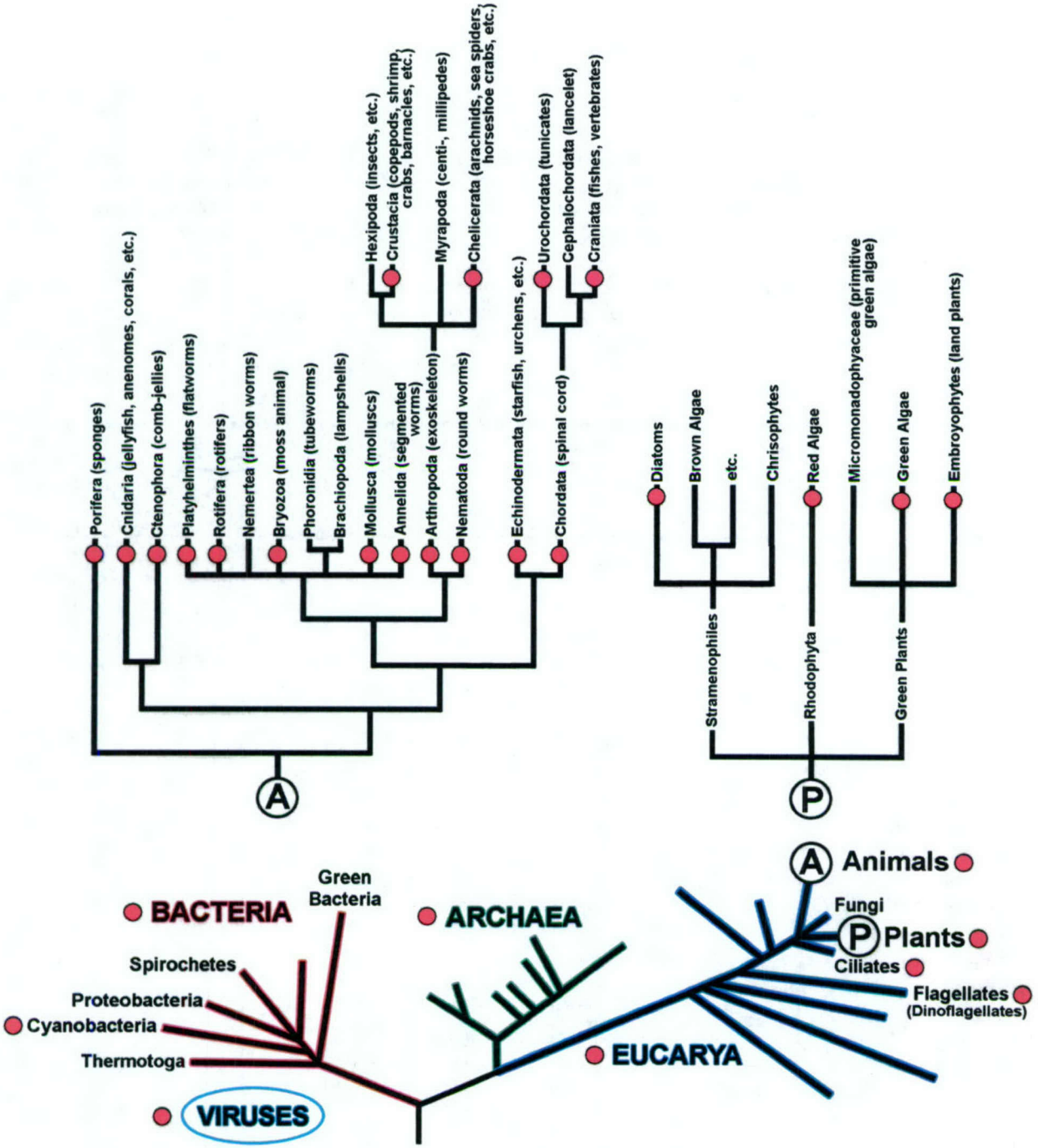


Figure 1. Phylogenetic tree illustrating the complex diversity of ballast water-borne organisms. Phylogenetic information obtained from Woese, 2000 and the Tree of Life Web Project (DR Maddison, Coord. Editor. http://tolweb.org/tree/). Groups encompassing ballast water-borne organisms indicated by ●, Smith, et. al, 1999; Wonham, et. al., 2001.

correctly, if sampling design was appropriate, and if the claimed treatment system effectiveness was consistent with the test program results. While it is not possible to address all test designs and assays here, this review identifies basic concepts and principals that should be included in virtually all test programs, as well as misconceived testing strategies that should be corrected. These points are discussed in the following section for each major taxonomic grouping.

Table 1. Breakdown of potential testing approaches and application by six BWT test programs. The results underscore the lack of general viability testing, particularly on the species level. The incorporation of the indicated key test categories is given by a Y (incorporated), N (not incorporated), I (inappropriate approach, typically a bulk measure interpreted as viability) or NA (not applicable). [A]Roderick, 2004; [B]Sutherland et al., 2001; [C]Waite et al., 2003.

[1] Bulk measures of chlorophyll for phytoplankton, used as surrogate for activity.

[2] Integrated "viability" over major taxa using ATP, PAM or other tentative viability measures.

[3] Autofluorescence enumeration, sometimes to species.

[4] Bulk measure of protein for <35 um and >35 um fractions, i.e. on mixed trophic levels.

[5]Development of resting stages in culture.

BWT Test Category	Testing Program						
	1[A]	2[A]	3[A]	4[A]	5[B]	6[C]	Total Yes
Zooplankton, Larvae							
Enumeration of total numbers	Y	Y	Y	Y	Y	Y	6
Resting stages analyzed	N	N	N	N	N	N	0
Population identified to genera/species	Y	Y	Y	Y	Y	Y	6
Viable organisms by population or group	Y	Y	Y	Y	Y	Y	6
Viable organism by species	N	N	N	Y	N	N	1
Instantaneous mortality	Y	Y	Y	Y	Y	Y	6
Delayed mortality	Y	Y	Y	Y	N	N	4
Phytoplankton							
Enumeration of total numbers	Y[1]	N	Y[1]	Y[1,3]	Y	Y[1]	6
Resting stages analyzed	N	N	N	N	Y[5]	N	1
Population identified to genera/species	N	N	N	Y[3]	Y	N	2
Viable organisms by population or group	I[1]	N	I[1]	Y[2]	Y	I[1]	2
Viable organisms by species	N	N	N	N	Y	N	1
Instantaneous mortality	I[1]	N	I[1]	Y[2]	Y	I[1]	2
Delayed mortality	I[1]	N	I[1]	Y[2]	Y	I[1]	2
Protozoa							
Enumeration of total numbers	N	N	N	Y	N	Y[4]	1
Resting stages analyzed	N	N	N	N	N	N	0
Population identified to genera/species	N	N	N	N	N	N	0
Viable organisms by population or group	N	N	N	N	N	Y[2]	1
Viable organism by species	N	N	N	N	N	N	0
Instantaneous mortality	N	N	N	N	N	Y[2]	1
Delayed mortality	N	N	N	N	N	N	0
Bacteria							
Enumeration of total numbers	Y	N	Y	Y	N	Y	4
Spores & resting stages analyzed	N	N	N	N	N	N	0
Viable organisms by population or group	Y	Y	Y	Y	N	Y	5
Viable organisms by species	NA	NA	NA	NA	NA	NA	--
Instantaneous mortality	Y	Y	Y	Y	N	Y	5
Delayed mortality	Y	Y	Y	Y	N	Y	5
Viruses							
Enumeration of total particles	N	N	N	Y	N	N	1
Viable viruses	N	N	N	Y	N	N	1
Instantaneous mortality	N	N	N	Y	N	N	1
Delayed mortality	N	N	N	Y	N	N	1

2.0 BALLAST WATER TREATMENT TESTING: STATUS AND NEEDS

Ballast water treatment testing is still in its early stages of development. Like the technologies to be tested, the proper approaches for determining treatment effectiveness on large, diverse, multi-taxa populations need to be developed. Test approaches need to take into account the variability of the populations to be assessed and the uncertainties in test results. Ideally, as in ecosystem assessment, testing could be simplified by working on functional groups rather than individual species. However, since ANS introductions are by individual species (not groups of species), this simplification is not generally valid unless the total organisms within a group are completely inactivated, removed or lowered to non-detectable levels. This lack of species-specific accounting of treatment effectiveness is one of the chief problems identified in the present test programs. However, the single major technical issue in BWT technology testing is the use of inadequate viability assays. In the case of BWT, loss of viability can result from the killing or inactivation of an organism or it may be simplified to mean that an organism cannot produce new organisms at any time post discharge. The definition of viability as "reproduction" results from the practical consideration that for a given species there will almost always be a minimum population density that will cause direct ecological damage. Even viruses must be able to replicate to cause ecological or public health problems.

The "perfect" BWT technology test program would evaluate the number and viability of each species before treatment, immediately after treatment, and again after a holding time, in order to assess moribund members of the population. This would allow for a quantitative determination of treatment effectiveness across the diversity of biological sensitivities found both within (e.g. eggs, larvae, adults) and between taxonomic groupings. In addition, after a number of these experiments it should be possible to determine indicator species, i.e., species within the plankton community that are the most resistant to treatment. As appropriate, these indicator organisms might then be the focus of future test programs in lieu of broad spectrum multi-species testing. At present, however, test programs have lacked necessary viability testing or have been hampered by the selection of viability assays that are either inappropriate or are applied in a manner which yields confusing results. Given the essential role of viability data in determining BWT effectiveness, the common test approaches and assays are reviewed and evaluated below for each major trophic group.

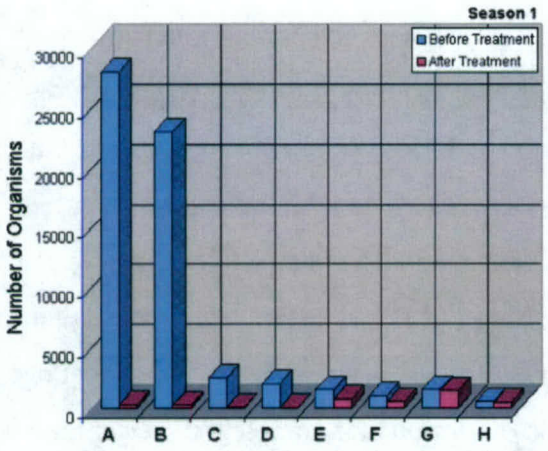
2.1 Zooplankton

Zooplankton (including larvae) tend to be both very taxonomically diverse and common within ballast water (Smith et al., 1999). In addition, zooplankton are relatively large and represent an important group with respect to bio-invasions from ballast water, and this has led to proposals for using a size standard that would require removal/inactivation of most zooplankters from ballast water discharges (Waite, 2002).

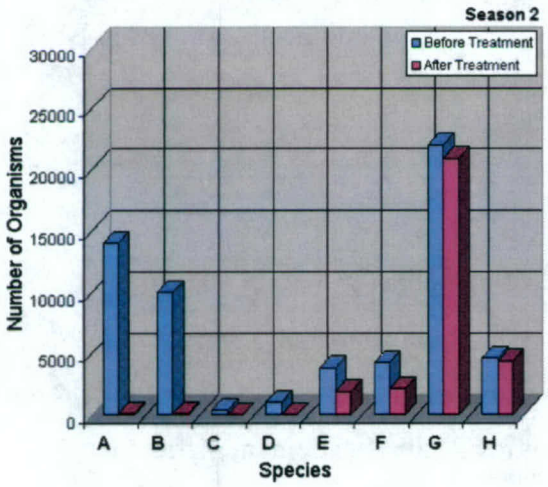
The best assays for addressing the effectiveness of a treatment system in preventing an invasion are those that directly assess, in as quantitative fashion as possible, each organism's viability upon discharge. For the zooplankton assemblage, a combination of assays (speciation, enumeration, and percent living versus dead) conducted over time can provide the needed viability information. The usefulness of the results will depend upon the extent that various genera/species are able to be distinguished and whether experimental protocols provide for adequate analysis of samples containing diverse numbers of many taxonomic types rather than procedures that analyze many taxa as a single group. The former approach allows for the assessment of the differential sensitivities of various taxa to the ballast water treatment and holding regimes.

In general, zooplankton are among the most appropriately evaluated groups in BWT test programs (Table 1). This stems in part from the ability of the investigator to enumerate and assess viability using relatively straightforward visual tests. Although genus- or species- level identification is generally available for zooplankton, most test programs rely upon general groupings such as copepods, polychaetes, etc. for quantification of response to treatment (Figure 1). This contrasts with ballast water transport studies conducted by the marine science community, which typically assay zooplankton to the species or genera level, and occasionally conduct larval grow-out studies for identification purposes (Smith, et al., 1999; Gollasch, et al., 2000; Olenin, et al., 2000).

In BWT testing, grouping of taxa can result in reasonably good evaluations of viability, but conclusions as to treatment effectiveness are confounded by not knowing if the BWT technology is equally effective across all taxa. This point is illustrated in Figure 2, upper panel, where the



Season 1					
Species	% Kill Rate	No. Before Treatment	No. After Treatment	% Remaining	Estimated % Kill
A	99	28000	280	1	99
B	99	23000	230	1	99
C	99	2500	25	1	99
D	99	2000	20	1	99
E	50	1500	750	50	50
F	50	1000	500	50	50
G	5	1500	1425	95	5
H	5	500	475	95	5
Total		60000	3705	6	94



Season 2					
Species	% Kill Rate	No. Before Treatment	No. After Treatment	% Remaining	Estimated % Kill
A	99	14000	140	1	99
B	99	10000	100	1	99
C	99	400	4	1	99
D	99	1000	10	1	99
E	50	3800	1900	50	50
F	50	4200	2100	50	50
G	5	22000	20900	95	5
H	5	4600	4370	95	5
Total		60000	29524	49	51

Figure 2. Effect of individual species resistance on determination of BWT efficiency, when an entire multi-species community is assayed as a single group. Season 1. Dominant species (A, B) highly sensitive to treatment, suggesting that treatment is effective even though minor resistant species (G, H) are nearly unaffected in numbers. Season 2. Over the seasonal cycle or at a different location, changing distributions of species with differing resistance to treatment can result in dramatic differences in quantified treatment effectiveness. This example illustrates the need for assessment of treatment effectiveness over seasonal cycles and differing locations and the limitation of expressing treatment effectiveness in terms of percentage.

dominant taxonomic groups (species A and B) are sensitive to treatment and are grouped with other taxa with varying levels of sensitivity. When treatment effectiveness is expressed as a percentage of grouped taxa (i.e., species A-H analyzed together), as is commonly done in testing programs, the treatment appears to be quite effective (94 percent kill). This result overshadows the true result that several of the more resistant species (E and F, and particularly G and H) escape the treatment in good shape when evaluated numerically. However, because they are relatively minor components of the population, their relatively high rate of survival is masked when expressed in terms of percentage. This is an important consideration when gauging BWT

effectiveness, since unequal effectiveness across taxa can mean that treatment to an apparently good total inactivation percentage (e.g. greater than 99 percent) may still result in certain treatment tolerant taxa remaining viable upon discharge and in sufficient numbers to cause an invasion. Additionally, seasonal changes in species composition can dramatically influence treatment system effectiveness if there is a significant change in dominance of resistant species (Figure 2, lower panel). At this early stage in the evolution of BWT testing, approaches that combine identification to species with species level viability determinations over seasonal cycles are necessary to provide a high quality approach. The exception is if complete physical removal or removal to below detection is achieved, such as might occur in certain size ranges in BWT systems which implement filtration.

2.2 Phytoplankton

Phytoplankton represent an important trophic group within ballast water. In addition to being potential ecological bio-invaders, certain taxa, such as toxic dinoflagellates, can have direct economic and human health impacts. Evaluating BWT effectiveness on phytoplankton presents difficulties, both in measurement and interpretation of results. Measurement difficulties are similar to other trophic groups in that the central focus needs to be species-specific viability/inactivation. However, phytoplankton present additional complications through their ability to repair themselves in light and to rapidly create new cells upon discharge (Sutherland et al., 2001). As a result, instantaneous viability assays may need to be paired with both holding time tests and post-treatment incubations. Unfortunately, while this approach has been used in BWT testing (Sutherland et al., 2001), it is not common (Table 1). Instead, bulk measurements such as extractable chlorophyll, which do not quantitatively reflect the effectiveness of treatment relative to cell viability, have been widely used.

Chlorophyll *a* has been used in a variety of test programs (Table 1) to assess viable total phytoplankton biomass. However, even if coupled with pheophytin (initial breakdown product) measurements, it does not quantitatively estimate viability, since the measured pigments can remain intact for indeterminate (but quite long) periods after cell death. The result of this persistence is consistent with the observed lack of a major effect of BWT on phytoplankton in recent shore-based BWT tests (Waite et al., 2003). These tests used chlorophyll *a* and

pheophytin as proxy measures of phytoplankton inactivation. The results showed little treatment effect on chlorophyll *a* and no change in pheophytin levels.

An important issue regarding the use of pigment assays as proxy measures of phytoplankton viability is that one can have phytoplankton cell disruption, an obvious lethal event, with the release of intact chloroplasts containing extractable chlorophyll, but the assessment would not register a change in viability. Pigment concentrations are not adequate to determine changes in phytoplankton numbers, since pigment levels are known to vary among species and with physiological state. This arises from the wide variation in the amount of chlorophyll per phytoplankton cell between species and even within species under varying environmental conditions. Using a pigment level approach, the loss of a few large cells (generally having high chlorophyll content) can appear to be a large percentage kill, even though more numerous smaller species remain viable. The result of using changes in extractable chlorophyll as an assay for viability/mortality greatly underestimates the effectiveness of the treatment system to control phytoplankton under ideal conditions (single species present or uniform kill rate across species) or to yield uninterpretable results when multi-species of variable sizes predominate. Unfortunately, multi-species assemblages are the norm within ballast waters.

Given the effects of toxic dinoflagellate blooms and the documented numbers of dinoflagellate cysts in ballast tank sediments (Hallegraeff and Bolch, 1991; Hallegraeff, 1998; Hamer, et al., 2000), it is surprising so few BWT test programs evaluate this sub-group of phytoplankton taxa (Table 1). The decision to omit evaluation of dinoflagellates from test programs reflects a choice in test design rather than a methodological constraint since the methods of identification and viability tests of this group are currently available and have occasionally been used in BWT tests (Sutherland et al., 2001). This decision is unfortunate since resting stages and cysts represent potentially good surrogates or indicators, but in order to support their use as surrogates, they need to be evaluated in comparison to other taxa.

Recent BWT test programs are exploring the use of proxy measures for determining phytoplankton viability through assay of the photosynthetic capacity. Pulse Amplitude Modulated (PAM) fluorometry has the potential for detecting stress in phytoplankton populations. However, because it is a bulk measurement and does not directly address effect on

potentially colonizable entities, it must be calibrated in some manner. It is not clear how a reduction in PAM fluorescence will be translated into a reduction in numbers of viable phytoplankton cells. In a pure culture, a reduction in PAM coefficients could be related to viability assays by Most Probable Number (MPN) assays or similar viability approaches. A direct correlation between an ANS-relevant parameter, viable number, and an easily measured physiological parameter would calibrate the PAM to viability. However, in the mixed phytoplankton assemblages within ballast water, PAM fluorescence parameters will be very difficult to interpret unless they are reasonably universal among species. The PAM approach may have promise, but at this point it is not clear how viability can be determined from this assay.

2.3 Protozoa and Bacteria

Bacteria in ballast water from polluted foreign harbors are a potential avenue for transmission of waterborne disease microbes, such as V*ibrio cholera*, enteric pathogens, *Campylobacter* spp., etc., into U.S. waters (e.g., McCarthy and Khambaty, 1994; Ruiz et. al., 2000). From the public health perspective, the classical viable count of select indicator organisms has been an assay that has been used for decades (Weiss and Hunter, 1939; Rompre, et. al., 2002). Growth on the surface of a nutrient medium (e.g., a nutrient agar medium or a filter placed onto the surface of a nutrient containing filter pad) into visible colonies, when enumerated, indicates those organisms that are able to reproduce and, hence, be a potential public health risk.

Similar approaches have typically been applied to assessment of the effectiveness of various ballast water treatment regimes on bacterial viability. For example, of the BWT test programs reviewed (Table 1), five of six assessed the general bacterial population in ballast water and all of these used the viable cell count assay. Though the bacterial viable cell count is useful for assessing treatment effectiveness, it is not as all encompassing as the zooplankton viability assessments, because only a minor fraction (e.g., 0.5-5 percent) of the bacterial population is quantifiable when compared with direct cell counts based upon staining and epifluorescence microscopy. This stems from the fact that only a fraction of the total bacterial population is able to grow on any given nutrient medium and, hence, be detectable. This restriction can be partially alleviated by the use of two to three broadly different nutrient media (e.g., a sugar based, protein

amino acid based, common central metabolite such as acetate-based media). In this approach, different fractions of the bacterial population are assayed by each media (different members of the population are able to grow on each medium), which allows for a broader assessment of the response to different ballast water treatment technologies. Similar responses of the different segments of the bacterial population to treatment would provide an indication of the degree to which the assayed population is representative of the entire population.

Fluorescent stain based measurements of bacterial numbers using epifluorescence microscopy have been used extensively in ecosystem studies as a convenient measure of total bacterial numbers. Because of its ease of use for environmental samples and the ability to preserve samples for later enumeration in the laboratory, epifluorescence direct cell counts have also found wide application in BWT testing programs (Table 1). The approach, however, provides little information on the critical test parameter, viability. Even in cases where lethal damage to the genome has been effected, there is not likely to be detectable changes in fluorescent properties, hence numbers of cells counted. In cases where the bacterial cells are physically removed or disrupted, fluorescent stains may produce useful data on BWT effectiveness, but treatments leaving the cells intact (e.g. UV, biocides, etc.) will tend to have their efficiency underestimated by this assessment approach.

Bacteria possess the ability to affect DNA repair and recover from potentially lethal damage to the genome. Most of the testing programs assessing bacteria (Table 1) are using both the viable cell count and direct cell counts using epifluorescence microscopy in holding experiments. It is possible that disintegration of lethally dosed organisms may be reflected in diminishment of cell numbers during holding periods, but this has not usually been observed. The primary difficulty is that without some way of specifically identifying the original population, changes in numbers will reflect "net" changes resulting from death and grow out. Death of larger organisms influences the nutrient field and can stimulate relatively rapid growth of remaining viable organisms, some of which may be minor contributors to the original population. An increase in bacterial numbers may not reflect recovery of affected organisms but growth of entirely different populations that may have statistically escaped the effects of treatment.

As research on available fluorescent vital stains continues, it is likely that this approach will address the viability issue for some functional groups. Vital stains that have been used to assess mortality in laboratory cultures may have application for the rapid assessment of viability in natural samples (e.g., Bernard et. al., 2001; Miskin, et. al., 1998). In these cases, living cells with intact cellular membranes stain differently than dead cells, allowing direct determination of mortality via epifluorescence microscopy. The primary limitation of such an approach will probably be the degree to which the complexity of the samples and differing physiological states of the organisms affect the ability of quantitatively distinguishing living from dead cells in practice. However, for this approach to work in BWT testing, it must be used in parallel with species identifications. Investigation of new techniques, such as Fluorescent In Situ Hybridization, should be encouraged in order to provide the necessary data and within the logistical constraints of shipboard testing.

Recent research has focused upon the application of molecular methods for both identifying microbes and assessing their viability status (Chandler, 2002; Keer and Birch, 2003). Presence of DNA (deoxyribonucleic acid) and r-RNA (ribosomal-ribonucleic acid) has proven not to be a good indicator of viability because of long persistence after cell death (Keer and Birch, 2003). Methods based on reverse transcription for quantifying messenger RNA (mRNA), a form generally thought to possess a short half life (minutes), showed initial promise as a viability indicator, but has proven in some cases to possess a poor correlation with viability because of persistence of some mRNA species for hours after cell death (e.g., Birch et. al., 2001). Methods based on transcriptional response to substrates might be a better assessor of viability than are methods requiring the decay of a short lived molecule where the end point is sometimes more difficult to determine (Esch, et. al., 2001).

Development of approaches for detecting viability in bacteria and protozoa is an area of intense basic research by investigators cognizant of the special needs of monitoring programs where time-consuming laboratory manipulation is not possible. Novel micro-array technology is presently being investigated and developed (e.g., Bavykin, et. al., 2001) for potential field application of the kind required by BWT programs, though at present discrimination between viable and nonviable microbes is not yet available. Potential exists in the future for an ability to

directly identify and quantify species of interest, perhaps at different trophic levels (e.g., bacteria, protozoa) and their physiological state.

2.4 Viruses

The concern for viruses in ballast water again stems from a public health perspective, including diseases such as infectious hepatitis, viral gastroenteritis, etc. Viral assays can be technically complex, usually because the host organisms that support viral growth can be difficult to culture. Most field studies involving viruses have centered upon the assay of the lytic bacteriophage where diluted samples are added to a plate covered with a host bacterium. Viral numbers are determined from the visible plaques which form where the host is lysed. Viral assays have not really become a part of routine BWT testing programs, as evidenced by the fact that only one of six programs illustrated in Table 1 has even attempted the measurement. Because the technical difficulty of a bacteriophage assay is no more complex than the bacterial viable cell count, the lack of viral testing in BWT programs is probably the result of not knowing what assay is best to use. It is of interest to note recent studies addressing the issue of a proxy measure for inactivation of pathogenic viruses such as the hepatitis A virus and poliovirus 1 (Nasser and Oman, 1999). This group found that a bacteriophage possessed longevity in various aquatic environments that exceeded the viral pathogens tested. It may be possible that bacteriophages, which are relatively easy to assay, may be reasonable proxies to some of the viral pathogens that are of public health interest.

2.5 Statistical Design and Sampling

Even using the highest quality methods and approaches for determining BWT removal or inactivation efficiencies, the results depend directly upon the use of proper experimental design to support statistical evaluation of the data. All BWT tests represent "experiments" in the purest sense. Each treatment system must be evaluated relative to untreated or control waters to determine the effects of the BWT system, as opposed to "natural" changes within the ballast water. This is particularly important, given that entraining coastal water within a ballast tank tends to result in the loss or gain of some taxa over time (Gollasch, et al., 2000; Olenin, et al., 2000; Smith, et al., 2000).

In addition, BWT technology effectiveness must be based upon the quantitative removal or inactivation of individual species or genera (as discussed above). Other than for physical removal or loss of organisms, viability testing or assays of the ability of the organism to propagate are required. At present, these types of assays require either grow-out incubations or direct visual assessment of freshly collected samples (Table 2). All of these types of assays could benefit from research to make them more amenable to the logistics of shipboard BWT testing. These assays are performed on the samples collected within the BWT testing experimental design in order to allow valid statistical testing of BWT technology effectiveness.

Table 2. Types of viability or inactivation tests suitable for determining the efficiency of BWT technologies. These types of assays are still limited by the need to evaluate certain functional groups on an individual species or genera basis.

Functional Group	Assays	Comments
Zooplankton & large animals	Species-specific counts with viability scoring	Viability as organism movement (motility, heart, cilia, etc), response to stimulation.
Phytoplankton	Species-specific counts immediately after treatment	Standard cell counts do not indicate viability
	Species-specific counts during grow out incubations	Change in numbers over time indicates viability
Protozoa	Species-specific counts during grow out incubations;	Change in numbers over time indicates viability
	Species-specific counts with viability scoring	Viability as organism movement (motility, cilia, flagella, etc),
Bacteria	Viable Plate Counts	Dilution series and growth into colonies
Viruses	Viable Plate Counts Phage Methods	Dilution series and growth determined by plaque formation
Cysts & Dormant Stages*	Quantitative microscopic assessment of germination in controlled incubations	Determination of fraction of dormant stages that are able to germinate

*These occur in several of the above groups and frequently represent a more resistant form of the organism than the active stages.

A critical element in the experimental design is the ability to provide proper controls with which to specifically compare the effectiveness of treatment. Ideally, control samples would be subjected to identical manipulations during the study, except for the specific treatment to be

measured. Additional, non-manipulated controls may be included to gauge "bottle or storage effect" alone but not to the exclusion of the directly comparable controls. Sample collection and manipulation should minimally affect the viability of the test organisms under study. Sampling points subjecting organisms to damaging shear forces should be avoided (e.g., forcing sample through sharp bends at high velocity; passage of sample through gear pumps for the purpose of sampling). If physical shear is unavoidable, such as passage through a ballast pump, treatment and control water streams should be identically subjected to the potentially damaging manipulation. Subjecting treatment and control streams to differing sources of shear or damage (e.g., passage of one stream through a single pump and the other stream through two pumps) will confound proper interpretation of the data. If differential manipulation is unavoidable because of the physical design of an installed treatment system, for example, then a second set of controlled experiments should be conducted to specifically address the effect of the additional source of damage. Studies in which control samples are killed nearly as completely as samples subjected to the treatment do not constitute a well-designed experimental program. Some mortality may be found in controls, as organisms retained in experimental test apparatus will likely be subjected to conditions significantly different from the natural environment. A well-designed test program will possess sufficient statistical robustness to readily distinguish with confidence the effects of treatment relative to controls that display modest mortality.

A good experimental design for BWT testing should include collection of both untreated and treated water from the ballast system with a time-course of samples to gauge both the instantaneous removal/inactivation on the different taxa and how these effects change with holding time (Figure 3). Given the need to account for the potential variability of incoming water and treatment efficiency during a single ballasting operation, replicate ballast tanks or smaller chambers should be filled and each sampled over time. The result is that for a single test of a single installed system, there needs to be a minimum of 2 treatments (control and BWT) x 2 tanks per treatment x 2 samples per tank per time point x 2 time points. This is a minimum of 16 samples to be assayed for viability of each species or taxonomic grouping. This alone represents a significant effort. Some programs, however, also conduct multiple assays on each sample.

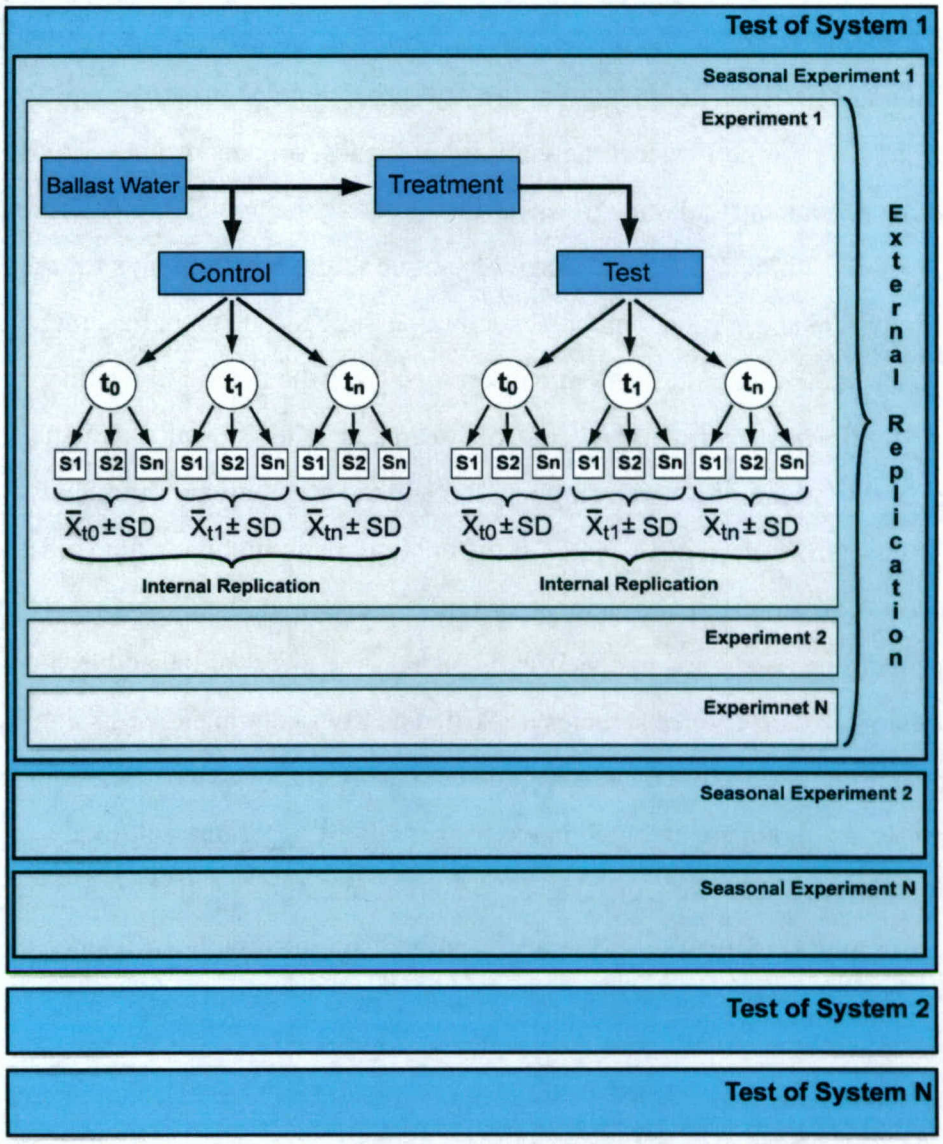


Figure 3. Generalized experimental design for BWT test programs. In typical experiments ballast water is pumped into reservoirs before (Control) and after passage through the treatment system (Test) and held. Samples are taken from the reservoirs in replicate (S1, sample 1; S2, sample 2; Sn, sample 3 or more) at various times, beginning with zero time (t0) immediately after the control and test reservoirs are filled. This provides an assessment of the immediate affect of treatment on the test organisms. Assessment of effects of holding (control) and delayed responses to treatment (test) are assessed by repeated sampling of both reservoirs at times t1 - tn. Results from the replicate samples (S1, S2, Sn) are analyzed statistically (e.g., determination of average ($\bar{x}$) and the standard deviation (SD)) to determine how well the sampling and analytical procedure measures the effect under investigation in a given experiment (Internal Replication) over time. Replication of the entire experiment a number of times (External Replication) provides a statistical measure of the consistency of a given treatment system under similar conditions. Replication of experiments over the seasonal cycle may be necessary because shifts in population structure may influence how well the treatment system performs (e.g., compare upper and lower panels in Figure 2). Seasonal studies will, hence, establish the robustness of system performance under the varying biological and physicochemical conditions a ship-based treatment system may normally encounter. Finally, because each ship installation is to some degree different, testing of different installations will ultimately establish the nominal performance standards of a given treatment system.

good practice, as it is frequently applied, it proportionately increases the number of assays within an experiment. Since analytical variation is generally small relative to the variation between replicate samples, programs need to evaluate the impact of increasing the number of samples and reducing the replication of assays, particularly if the assays are complicated or time consuming. This shift in effort will still allow for statistical evaluation of treatment effectiveness and the role of holding time. In addition, the "savings" in effort can then be allocated toward collecting more samples within each test tank on each time point or more importantly in establishing more test tanks within each treatment (since the largest variability is typically between replicate tanks). Due to system/site specific variability of individual test platforms and BWT systems, it is not possible to generically define the appropriate number of replicate tanks and samples that will be sufficient to allow discrimination of BWT efficiency. Instead, each test program needs to conduct an evaluation of the allocation of replication (i.e., effort) to improve the testing of the main experimental effect, the difference between control and treated ballast waters.

An additional complication in testing design is based upon the use of a single ballasting operation and single BWT technology unit to run a test. In fact, this really represents a single experiment with no replication at the level of the treatment system and captures virtually none of the natural variation in coastal and fresh waters or even the usually significant seasonal variation at a single site (Zhang and Dickman, 1999). The typical approach to dealing with this issue in almost all of the six test programs represented in Table 1 has been to conduct multiple experiments, i.e., multiple runs (sometimes at different sites) over time. While this is both practical and greatly improves the results, the issue of testing only a single BWT unit on a single platform still remains. This is especially problematic when one considers that there are significant ship-specific differences in the handling of ballast water even if the sample BWT technology is employed. With a single BWT unit being tested, it is possible to statistically test the effectiveness of that unit, but multiple units need to be tested to evaluate the effectiveness of the technology as a whole (Figure 3). One approach for bringing the need for proper experimental design together with the practical realities of the maritime industry is to conduct intensive shore-based test facility studies of replicate BWT units over time. If the results are adequate, then conduct shipboard testing (at a reduced level) on a specified number of vessels. This phased approach should support full evaluation of a technology to determine if wide-scale installations are warranted.

3.0 BWT TECHNOLOGY EVALUATION

A successful testing approach, based upon the experimental design requirements and logistical realities of BWT technology evaluation, could be achieved by combining the programs being proposed by the USCG and its agency partners. One proposal is to continue BWT test programs either conducted independently or in concert with sophisticated shore-based test facilities, such as has been established in Florida (Waite et al., 2003) and the NRL facility currently under construction, or through the USCG's Shipboard Technology Evaluation Program (STEP). Test facilities could be linked to the USCG/USEPA effort through the Environmental Technology Verification program, whose goal is to accelerate the evaluation process to get environmentally important technologies implemented as soon as possible. Following initial demonstrated BWT effectiveness, the USCG could then implement the Shipboard Technology Evaluation Program that would provide test data on a BWT technology from multiple vessels. Given the environmental scope of the invasive species problem, it appears that one of the best approaches for thorough BWT technology evaluation will require integration of data from multiple initiatives. This approach will provide both the internal and external replication required for proper evaluation, while not introducing unreasonable delays in BWT implementation. Unlike some new technologies, the implementation of ineffective BWT technologies causes "harm" by not stemming the continued likelihood of bio-invasions through ballast water discharges. The proposed multi-phase approach to full approval reduces the potential for widespread implementation of systems that are ineffective, while increasing the likelihood that effective technologies will not be rejected.

4.0 BWT TESTING NEEDS

While it is clear that species-specific viability testing is needed to properly evaluate the efficacy of BWT technologies, there is a need for simplified approaches and new techniques, particularly rapid assays. These needs would be facilitated by research initiatives aimed at developing rapid viability tests or approaches that allow calibration of simplified approaches. The development of new and/or rapid viability tests should include an examination of proxy measures of viability. Some of these measures are common to aquatic science, for example adenosine tri-phosphate (ATP) or its relative, electron transport system (ETS). However, existing ATP related approaches will require significant testing and development before they will be useful for quantitative viability testing. The issues of BWT testing, logistics, limited laboratory facilities at sea, and cost are all common issues in biological oceanographic research. Viability assays which can be applied to samples at sea and preserved for assay ashore, for example stains (Bernhard et al. 1995, Bernhard 2000) or fluorescent tags, will be of particular value, both for present testing by multidisciplinary teams and in future monitoring of ship-based systems.

Similarly, research into potential surrogate or indicator species, both endemic and for introduction in test bed evaluations, will serve both to reduce evaluation costs and allow for the necessary sample replication. Research into surrogate and indicator species must be conducted so that it calibrates the most tolerant taxa to each of the less tolerant or sensitive taxa, for each new treatment technology. This may require that different indicators or suites of indicators be employed based upon the specific mechanism(s) used in different technologies, although it is unlikely that more than a few indicators will need to be developed. In the long-term, with continued industrial BWT R&D testing, the establishment of test bed facilities and directed research into new viability assays and test approaches, it is almost certain that significant streamlining of testing, with associated reductions in cost, will occur. However, what is needed in the short-term is directed research to facilitate this evolution, which will benefit both those focused upon ballast water treatment and the wider marine research community.

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