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The case for using the Most Probable Number (MPN) method in ballast water management system type approval testing

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ABSTRACT

Recently, the U.S. Coast Guard (USCG) rejected the Serial Dilution Culture-Most Probable Number (SDC-MPN) method for enumerating viable phytoplankton cells in ballast water discharge as an alternate to their prescribed method — the Environmental Technology Verification (ETV) Protocol. This method distinguishes living from dead organisms using vital stains and motility. Succinctly, the USCG position has been that the ETV Protocol is a reliable and repeatable efficacy test and the SDC-MPN method is not. New evidence and an expanded consideration of published research supports a fundamentally different assessment.

A peer-reviewed quantitative evaluation of ETV vital stains for 24 species of phytoplankton has conclusively established that the ETV Protocol, even with observations of motility, is not reliable for all species. In contrast, published results suggest that errors in the method were small for the limited number of locations studied to date. It is possible that the communities tested in these were dominated by species that can be classified accurately using vital stains. Even so, it must be acknowledged that the reliability and accuracy of vital stains is untested for thousands of species of phytoplankton.

Introduced in 1951, the SDC-MPN method for phytoplankton is an established approach for use with multi-species communities. As applied to ballast water testing, SDC-MPN is much less vulnerable to methodological uncertainties than has been assumed. Notably, all species of phytoplankton need not be cultured in the conventional sense. Rather, a single viable cell in a dilution tube need grow only enough to be detected — a requirement known to have been met by otherwise uncultured species. Further, delayed restoration of viability after treatment with ultraviolet radiation (UV) is not a problem: organisms repair UV damage quickly or not at all, consistent with the assumptions of the test.

Two critical methodological failures could compromise protection of the environment in ballast water testing: living organisms that do not stain or move, and viable organisms that do not grow to detection in the MPN cultures. These can be assessed with complementary measurements, but importantly, the relative protection of each method can be evaluated by comparing counts of living cells from the ETV Protocol with counts of viable cell from SDC-MPN in untreated samples. Available evidence provides no basis for concluding that either method is consistently less protective. However, as applied in ballast water testing, the statistical estimate of MPN is less precise. On this basis, SDC-MPN is worse for a single test. But, counter-intuitively, it is more protective of the environment when five consecutive tests must be passed for type approval, because the likelihood of one false rejection out of five tests is higher and five false passes would be exceedingly rare. Addressing only the science, we conclude that both the ETV Protocol and the SDC-MPN method, though imperfect, are currently appropriate for assessing the efficacy of ballast water management systems in a type-approval testing regime. In closing, we show proof of concept for a rapid assay of viability, benchmarked against SDC-MPN, that could be well suited for routine assessment of treatment system performance.

Keywords: Ballast water management, MEPC, motility, USCG, viability, vital stains

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1. Introduction

In a final action that was explained on their web site (Coast Guard Maritime Commons, 2016a) and documented in letters of denial (Fagan, 2016), the US Coast Guard (USCG) rejected the Most Probable Number (MPN) method for enumerating viable phytoplankton cells in ballast water discharge as an alternate to their prescribed method — the Environmental Technology Verification (ETV) Protocol (ETV, 2010). This method distinguishes living from dead organisms using the vital stains fluorescein diacetate (FDA) and 5-chloromethyfluorescein diacetate (CMFDA), with motility as a confirmatory criterion (Steinberg *et al*., 2011). As summarised in their blog (Coast Guard Maritime Commons 2016b), the USCG position has been that the ETV Protocol is a reliable and repeatable efficacy test and the MPN method is not. This assessment was confirmed by Rear Admiral Paul Thomas in his April 14, 2016 congressional testimony: “[There] is a reliable, repeatable efficacy test to determine if something is dead. There is not a reliable, repeatable efficacy test to determine if they have been rendered harmless” (U.S. Congress, 2016, p. 13).

More recently, the United States recommended to the Marine Environment Protection Committee (MEPC) of the International Maritime Organization (IMO) that a general version of the FDA/CMFDA + Motility Method be accepted and communicated via an appropriate circular (United States, 2016b). In turn, they recommended that acceptance of an MPN Dilution Culture + Motility Method (submitted by Denmark & Norway, 2016) be deferred pending further documentation of method validation, which the US judged to be unclear. All recommendations and discussions apply to enumeration of viable organisms in the 10 - 50 µm size range.

Attention is now appropriately focused on the validation of each general method for assessing the efficacy of ballast water management systems. Importantly, in its recent review of G8 guidelines (MEPC 70/WP.5), the IMO has provided criteria for the assessment: methods should enumerate *viable organisms* (i.e., those able to successfully generate new individuals to reproduce the species) using *measures that are appropriate to the ballast water treatment technology tested*. As presented here, a review of recent evidence and an expanded consideration of published research provides support for concluding that both the FDA/CMFDA + Motility Method and the MPN Dilution Culture + Motility Method, though imperfect, are currently appropriate for assessing the efficacy of ballast water management systems in a type-approval testing regime.

2. The FDA/CMFDA + Motility Method

The general method

Presented as a general method for acceptance and communication by the IMO, the FDA/CMFDA + Motility Method (United States, 2016c) is a modification of the ETV Protocol that was described and validated by Steinberg *et al*. (2011). In particular, the pre-concentration of samples on a 10-µm filter has been omitted and microbeads are now added to samples as a size reference. These two changes align the proposed method with no-filtration practices recommended by Denmark & Norway (2016). Strictly, validation of the ETV Protocol would not apply to the FDA/CMFDA + Motility Method proposed by the United States because of methodological differences, but the principles underlying each are the same, so validation of the ETV method is relevant. The same reasoning applies to the variants of the MPN Dilution

Culture-Motility Method (Denmark & Norway, 2016): as suggested by the United States (2016b), a generic, base method can be validated and approved, with specific standard operating procedures validated by individual test organisations.

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Validation of the ETV Protocol

According to the US submission (and also a USCG-commissioned review: Drake *et al*., 2016), the quantitative validation of the ETV Protocol, including the testing of method assumptions, is described in one peer-reviewed publication (Steinberg *et al*., 2011). This study of protists in the 10 - 50 µm size class consisted of preliminary observations of laboratory cultures and natural assemblages followed by quantitative tests on natural assemblages from four locations (Table 1).

Table 1. Validation of the general FDA/CMFDA + Motility Method submitted by the United States for consideration by the IMO MEPC (United States 2016c). The study by Steinberg et al. (2011) validated the closely related ETV Protocol, which includes a pre-concentration step that is not in the general method. Utility of the ETV Protocol was demonstrated by Drake et al. (2012) — no problems with implementation were reported by two test facilities. However, that study did not include the live-vs.-dead comparisons in the original validation. The study by MacIntyre and Cullen (2016) is not referred to by the U.S. as a validation, but the experimental design is consistent with their recommendation to conduct supplemental validation "using protists maintained in an active growth phase under culture (positive controls) and protists that have been heat-killed (negative controls)." Cell counts for individual samples in the ETV Protocol validation were reported by Steinberg et al. (2010).

Validation study	Diversity	Comparisons	Measurement	Total cells measured	Validation for individual species
U.S. ETV Protocol (Steinberg et al. 2011)	Natural assemblages 20 samples 4 U.S. locations No fresh water 5 May – 2 Oct	Stained with FDA+CMFDA: No treatment "live" Heat treated "dead" Cold-treated "dead" Stained vs. unstained "dead"	F+ or F– and M+ or M– assessed by microscopist	6,703	No
Stains as live-dead indicators (MacIntyre & Cullen 2016)	24 species 7 Divisions Fresh water & marine 3-5 fully independent replicates	Stained (FDA, CMFDA, FDA+CMFDA) and unstained: Growing, verified "live" Heat treated, verified "dead"	Calibrated flow cytometer (F_{green}: dynamic range of 10^6)	9,713,986	Yes

The base assumption of the FDA/CMFDA + Motility Method is that after staining, cells with detectable green fluorescence (F+), as observed by a microscopist, are "live". As a back-up, cells without detectable green florescence (F–) are observed for motility. If they move (F–/M+), they are also classified as "live", otherwise (i.e., F–/M–), they are considered dead. Steinberg *et al*. (2011) validated the FDA/CMFDA + Motility Method by observing organisms that had been killed; for those cells, F+ represents a false positive error. (Killed cells were uniformly M–.) Tests were conducted on a total of 20 samples from four locations in the

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United States; in total, 6,703 cells were examined (Table 1). Generally, false positive errors in the method were low, and very few moving cells were F–, suggesting low rates of false negative error for the stains. The method was judged to be, “a robust, powerful tool that can be optimized for the species present at each location” (Steinberg *et al*., 2011).

One aspect of the validation was inconclusive, however. As recognized by Drake *et al*., (2016), living organisms that are classified as F–/M– may represent undetected false negative errors: living phytoplankton that don’t stain and can’t or don’t move under the microscope (see MacIntyre & Cullen, 2016). This uncertainty cannot be resolved using the existing ETV Protocol validation because there is no guarantee that all untreated organisms in natural assemblages are alive. Consequently, both the Drake *et al*. (2016) review and the US submission to MEPC recommend supplemental validation of FDA/CMFDA + Motility Method “using protists maintained in an active growth phase under culture (positive controls) and protists that have been heat-killed (negative controls).” By the same reasoning, these prescribed laboratory studies would validate the base assumptions of the MPN method.

Validation of the accuracy of FDA+CMFDA as vital stains

Recently, the base assumptions of the FDA+CMFDA method were tested in a comprehensive, quantitative and replicated peer-reviewed study (MacIntyre & Cullen, 2016). The experimental approach — observations on actively growing and heat-killed cultures — was entirely consistent with the U.S. recommendation, but the study differed in using a flow cytometer to make quantitative measurements of green stain fluorescence, eliminating uncertainty due to a microscopist’s perceptions. MacIntyre and Cullen (2016) quantified fluorescence in 9.7 million cells compared to the 6.7 thousand cells examined by Steinberg *et al*. (2011) (Table 1). The results were clear: FDA + CMFDA stains worked as assumed for some species but not for others. Applying a criterion of < 10% error in distinguishing demonstrably living from dead organisms, the stains were accurate for 10 of 24 species examined. For four of the species, dead cells stained more strongly than living cells. Contrary to indications that a particular taxonomic group, dinoflagellates, was more prone to misclassification using stains (Steinberg *et al*., 2011; United States, 2016c), the study of 24 taxonomically diverse species in culture showed no relationship between staining patterns and taxonomy (MacIntyre & Cullen, 2016).

Given these recent results, it can be confidently and unequivocally stated that the FDA/CMFDA + Motility Method is not reliable and accurate for all species of phytoplankton. But the ETV Protocol validation and at least one other published study (Adams *et al*., 2014) suggest that errors in the vital stains method were small for the limited number of locations studied to date. It is possible that the communities tested in these were dominated by species that can be classified accurately using vital stains. Even so, it must be acknowledged that the reliability and accuracy of vital stains is untested for thousands of species of phytoplankton. This same criticism has been directed at the MPN method (Fagan 2016, p. 21).

3. The MPN Dilution Culture + Motility Method

The need for an alternate method

Unlike vital stains, which test for membrane integrity and the presence of certain biomolecules — indicators of life — the MPN Dilution Culture Method (Serial Dilution Culture - MPN method, SDC-MPN: Cullen & MacIntyre, 2016a) tests directly for viability — the ability to reproduce — thereby ensuring alignment with IMO G8 guidelines. The method is used in ballast water testing because treatment with ultraviolet radiation (UV) renders organisms harmless by damaging DNA and preventing reproduction without necessarily killing

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organisms outright. Consequently, SDC-MPN, not vital stains, is appropriate for testing ballast water treatment with UV. This is recognised explicitly for treatment of drinking water (USEPA, 2006). Although the USCG has decided that the ETV Protocol can and should be used for assessing ballast water treated with UV (Coast Guard Maritime Commons, 2016a), recently, the United States stated that the method is not appropriate for UV systems: “This [FDA/CMFDA + Motility] method would be appropriate for ballast water treatment technologies designed to remove or kill organisms, rather than render living organisms non-reproductive.” (United States, 2016a, p. 3).

Because the SDC-MPN method is designed to enumerate only viable photosynthetic organisms, in the MPN Dilution Culture + Motility Method, organisms that lack detectable photosynthetic pigment (designated “heterotrophs”) are enumerated under the microscope, using motility as a criterion for viability. As reviewed by Denmark & Norway (2016), this can lead to large overestimates of viable heterotrophs after treatment with UV (errors that are protective of the environment), and underestimations to the extent that viable non-photosynthetic organisms do not move. Although the heterotroph method has some uncertainties (Drake *et al*., 2016), the SDC-MPN method has generated the most controversy (Cullen & MacIntyre, 2016b), and it will be the focus of this discussion.

SDC-MPN: a well-described, general method

Introduced more than 60 years ago by Wyn Knight-Jones (1951), the SDC-MPN method for phytoplankton is an established approach for use with multi-species communities. Significantly, the most abundant organism in Knight-Jones’s dilution cultures was previously undescribed, demonstrating that prior knowledge of specific culturing requirements was not needed. The method is based on dilution culture, a technique used on phytoplankton for nearly a century (Allen, 1919) and Most Probable Number calculations, initially developed for interpreting results of sanitary analyses (McCrady, 1915). The principles of the numerical MPN method (Cochran, 1950) are the same, whether for bacteria or phytoplankton. The SDC-MPN method has been reviewed in UNESCO reference works on phytoplankton methods (Throndsen, 1978: Andersen & Throndsen, 2003). More recently, Cullen and MacIntyre (2016a) examined it in the context of ballast water testing. They, and Denmark & Norway (2016), respond to criticisms of the method and discuss sources of uncertainty.

Validation of the SDC-MPN base method

Consistent with recommendations for validating the base assumptions of the FDA+CMFDA method (Drake *et al*., 2016; United States, 2016c), the basis of the SDC-MPN method was validated using actively growing and heat-killed cultures (MacIntyre & Cullen, 2016). All five of the species tested were accurately classified using SDC-MPN (Figure 1) whereas fewer than half of 24 species tested with vital stains were accurately classified using FDA+CMFDA. The five species tested using SDC-MPN had very high error rates, >94%, when classified with FDA+CMFDA (MacIntyre & Cullen, 2016).

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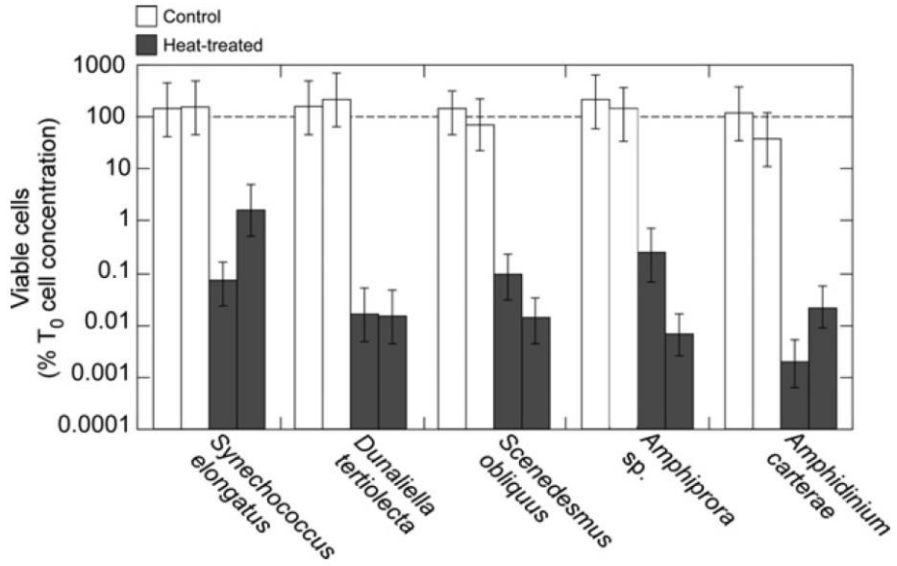
Figure 1. Validation of the base SDC-MPN method (Figure 7 from MacIntyre and Cullen 2016). Viable cell concentrations were estimated using replicated SDC-MPN measurements. Replicated experiments are shown as separate columns. Error bars are the 95% confidence intervals. In actively growing cultures (Control), estimates of viable cells did not differ significantly from counts of total cells (dashed line); similar results were obtained for ten other species. In turn, SDC-MPN accurately indicated >99% loss of viability in heat-killed cultures. The base method for SDC-MPN was thus validated for 5 of 5 species. In the same publication, the base FDA/CMFDA method was shown to accurately distinguish live from heat-killed cells in, at best, 10 of 24 species. This figure is used under Creative Commons Attribution License.



Addressing technical criticisms of SDC-MPN

Key concerns about the SDC-MPN method have been presented by the USCG (e.g., US Coast Guard, 2012; Coast Guard Maritime Commons, 2016b) and responded to in various ways, including manufacturers' applications and appeals (addressed by Fagan, 2016), a peer-reviewed examination with follow-up (Cullen & MacIntyre, 2016b; Cullen and MacIntyre, 2016a) and a recent submission to MEPC (Denmark & Norway, 2016). The evidence suggests that, as applied to ballast water testing, SDC-MPN is much less vulnerable to methodological uncertainties than has been assumed:

- Notably, all species of phytoplankton need not "culturable" in the conventional sense; for accurate enumeration, a viable organism in a culture tube needs to divide only enough times to be detected. Species are known to have this capability even though they are difficult to maintain indefinitely in laboratory culture (Throndsen, 1978).
- Specific culturing requirements need not be known in advance. The method has been used routinely to isolate previously undescribed organisms (reviewed by Cullen & MacIntyre, 2016a).
- Delayed restoration of viability after treatment with UV, identified as a concern (Coast Guard Maritime Commons, 2016b) is not a problem: scientific evidence accumulated over decades indicates that microorganisms repair UV damage quickly or not at all, consistent with the assumptions of the test.

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Still, the SDC-MPN method is subject to several sources of error, including species that grow too slowly or not at all under the conditions provided, cells smaller than 10 µm contaminating the samples and inflating estimates of viable organisms, potential grazing in culture tubes leading to underestimation of viable phytoplankton, and the presence of multicellular colonial forms that can complicate enumeration of single organisms within a size range. Denmark & Norway (2016) evaluate each source of error in the context of ballast water testing, and provide a corresponding list of sources of error in the FDA/CMFDA + Motility Method, with suggestions for best practices in the further evaluation of each method. They emphasise that despite the limitations of the two methods, both "exceed in their reliability many of the available biological test methods and can therefore be used for evaluation of concentration of viable organisms in the 10 to 50 µm size class."

4. Comparing methods for equivalent protection of the environment

The evidence reviewed here does not support the binary assessment of testing methods — that one is reliable and repeatable, the other is not. Rather, there are reasons to argue that both methods have been validated (Table 1; Figure 1) and shown to be useful, though subject to limitations (Denmark & Norway, 2016). Further evaluation of methods can focus on statistical uncertainties, particularly in the context of ballast water management system type approval. The central question is whether or not the methods provide equivalent protection. Two statistical measures are generally used to compare methods. *Precision* represents scatter in the estimate — the spread of data around its mean, e.g., due to random measurement error. *Accuracy* is a measure of systematic error (bias) — the variation of the mean of the measurements from the true value.

Precision

The FDA/CMFDA + Motility Method is subject to random error in the counting of viable cells, which is very strongly a function of the total number counted (Venrick, 1978; Edler & Elbrächter, 2010). When 100s of cells are counted, as recommended by Denmark & Norway (2016), errors (± 95% confidence limits) are less than 20%. This measurement uncertainty is less that for typical MPN estimates, which are well known to have relatively wide confidence intervals (Knight-Jones, 1951). But when concentrations of viable cells are well below the discharge standard of 10 per ml and — as specified by the US and Denmark & Norway — samples are not concentrated, fewer cells could be counted in the specified time interval and precision could be worse than for MPN.

Assuming that systematic error is not an issue, a more precise test is clearly better because inappropriate approval or rejection due to random measurement error is less likely. But a typical type approval trial requires five consecutive successful tests, and the implications of precision are much different: counter-intuitively, random errors are protective of the environment. Consider a test that, though accurate, is so imprecise that it returns a false "pass" (viable cells < 10 per ml, even though the mean concentration is > 10 per ml) 50% of the time (i.e., $p = 0.5$). This would seem an unacceptable risk to the environment. But in type approval, five successive passes are required for approval and the probability of the unwanted false result would be 0.5 to the fifth power, or 3.1%. Simply, the consecutive-test rule ensures that ballast water management systems are highly unlikely to pass type approval inappropriately due to imprecision in the method. Complementary calculations (J.J. Cullen, unpublished) show further protection of the environment from the 5-test regime: to avoid false rejection of a compliant system due to imprecision in the measurement, the mean concentrations of viable cells must be lower than the regulatory standard to ensure a correct approval. We conclude that neither method presents an inherent risk to the environment due to random error in the measurements.

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Accuracy, equivalency and protection of the environment

Denmark & Norway (2016) listed sources of systematic error (overestimation and underestimation) for both the FDA/CMFDA + Motility and MPN Dilution Culture + Motility methods. They point out that overestimations are conservative (and thus underestimations bring risk) when discharge concentrations are measured, but the opposite is true for control samples that must be measured to assess test validity. For consistency with established arguments (ETV, 2010; US Coast Guard, 2012), we will consider overestimations to be conservative when relating systematic errors to protection of the environment (Cullen & MacIntyre, 2016b).

Two critical methodological failures lead to underestimation and risk to the environment: living organisms that do not stain or move, and viable organisms that do not grow to detection in the MPN cultures. It is important to quantify these errors. However, as explained by Drake et al. (2016), there is no independent reference method to determine the true concentration of viable organisms in natural communities of protists, so it is not possible to measure directly the *accuracy* of the FDA/CMFDA + Motility or MPN Dilution Culture + Motility methods. But, as they point out, the methods can be compared for *equivalency*, the difference in their means. And because higher counts are more protective of the environment in the analysis of ballast water discharge, the comparison can be interpreted in terms of equivalent protection. Simply, the method that produces the higher estimate is by definition more protective of the environment. If neither method produced consistently lower counts, it would be evidence of equivalent protection of the environment. The comparison would have to be made on untreated samples, because it is already established that vital stains overestimate the number of reproductive organisms in ballast water treated with UV (Wright & Welschmeyer, 2015; United States, 2016a).

A comprehensive comparison of parallel counts from the FDA/CMFDA + Motility and MPN Dilution Culture + Motility methods is not available. Five of five trials reported by Molina *et al*. (2016) yielded higher counts for MPN + Motility (indicating that MPN is more protective), but calculation of exact ratios is complicated by a mismatch between chosen dilutions and initial concentrations that does not influence the following conclusions. Both the DHI Maritime Technology Evaluation Facility and NIVA's Ballast Water Testing Facility (see Denmark & Norway 2016) submitted comparison data to the ETV Program in the United States — 73 and 32 untreated samples, respectively. A comprehensive analysis would require careful consideration of methodological details. But a broad characterization is possible: of the 110 comparison samples from three laboratories, counts of viable cells from the MPN-based method were greater in 58, and FDA/CMFDA-based counts were greater in 52. The mean of the log-transformed ratio of MPN counts/Stains counts was not significantly different from zero, indicating that there was no consistent difference between methods in counts of viable cells. This preliminary analysis does not prove equivalent protection, but it does support the statement that available evidence provides no basis for concluding that either method is consistently more, or less, protective.

5. Conclusion

Based on scientific evidence presented here, we conclude that the general methods underlying the FDA/CMFDA + Motility and MPN Dilution Culture + Motility methods, though imperfect, are currently appropriate for assessing the efficacy of ballast water management systems in a type-approval testing regime. Because the FDA/CMFDA + Motility Method does not accurately assess the effects of UV treatment on reproduction, both methods should be available for use if efficacy tests are to be, as specified by IMO, "appropriate to the ballast water treatment technology being tested."

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