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To: Ballast Water Stakeholder Community

Regarding: US Coast decision on MPN test assay for 10-50 μm protists

According to the International Maritime Organization (IMO) G8 Convention, the objective of a successful ballast water management system (BWMS) is "... to remove, render harmless, or avoid the uptake or discharge of harmful aquatic organisms" which might otherwise lead to the spread of aquatic invasive species under routine ballast water discharge practices. The recent decision by the US Coast Guard to consider the most probable number (MPN) method inappropriate for the regulatory numerical evaluation of autotrophic protists (phytoplankton) in ballast water is surprising since:

- 1) The MPN method is already approved for measuring the regulated levels of the potential pathogenic bacteria, *E. coli* and *Enterococcus*, in ballast water Type Approval tests.
- 2) The MPN method numerically evaluates the ability of phytoplankton to grow under optimized culture conditions, thus closely mimicking the core invasion process that occurs in nature, e.g., organism growth.
- 3) The MPN method is substantially more sensitive (lower limit of numerical detection) than the current microscope-based counting technique, which utilizes an enzyme-based fluorescent tag, fluorescein diacetate (FDA) and/or chloromethylfluorescein diacetate (CMFDA).
- 4) The MPN technique is free from the 'false positive' live counts that can be associated with numeric FDA/CMFDA counting, where residual enzyme activity can be found in dead/dying cells, even though those cells have no capacity for growth.
- 5) The MPN technique appropriately evaluates the efficacy of biocidal treatments, such as Ultraviolet (UV) irradiation, which inactivates growth capacity in microorganisms through DNA damage, without rendering an immediate condition of death.

The last point above, in reference to UV treatment, is particularly significant since it is well known that UV-treated organisms in ballast water can yield numerous false-positive 'live counts' in FDA tagging procedures, such that regulatory numeric levels are exceeded (erroneously). In a recent analysis of 81 paired measurements of UV-treated ballast water samples by 1) FDA-based counting and 2) MPN incubation protocol for 10-50 μm phytoplankton, 89% of all tests evaluated by FDA exceeded numeric ballast water discharge standards (BWDSs) while 100% of all tests of the same UV-treated ballast water passed

BWDSs on the basis of MPN assays (Wright and Welschmeyer, 2015). Moreover, most MPN estimates of UV-treated ballast water yielded <0.06 live cells per mL, which is almost an order of magnitude lower than the limit of detection achievable with FDA counting protocol.

The argument given by USCG in the denial of MPN as an alternative assay for FDA counting is that the ‘live/dead’ status is not measured explicitly; they cite issues on the interpretation of ‘live’ vs. ‘viable’ cells. This position is tenuous since the live/dead status of all microorganisms, in general, is confusing and often linked to the unique chemical characteristics of the measurement assay itself; not to ‘death’, nor ‘life’, per se. The early, seminal work on FDA (Rotman and Papermaster, 1965) introduced the FDA reagent as a viability indicator for mammalian cells capable of ‘cloning’, e.g., growing. Haugen et al (1995), in the original US Patent description for CMFDA, described the reagent as a unique tracer for viable cells, while the review by Davey (2011) refers to colony growth and cell proliferation as the ‘*gold standard*’ for the determination of viable cell concentrations. Thus, in microbiology, the distinction between ‘viable’ and ‘live’ is somewhat muddled, and the terms are often used interchangeably. Davey’s 2011 review, “Life, Death, and In-Between: Meanings and Methods in Microbiology“ provides ample context to question the USCG argument which cites specific (but ambiguous) language (viable vs. living) as a solid platform on which to approve/disapprove assays for biological efficacy in ballast water testing.

The objective in the design and testing of ships’ ballast water management systems (BWMS) is to abate the spread of aquatic invasive species. The MPN technique operates on the principle of careful analysis of growth characteristics of potentially invasive organisms; it is aligned with the actual mechanism of invasions – organism growth. This fact, together with the acknowledged false-positive errors of FDA/CMFDA tagging, suggests that a re-evaluation of the USCG decision on MPN testing protocols is warranted.

Sincerely,

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