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MPN Dilution-Culture Method as an alternative method for measuring treatment of organisms in the 10-50 um size category

Background. The United States Coast Guard published on December 14, 2015, its opinion that the Most Probable Number method (or MPN method) is not considered an equivalent alternative to the testing methods prescribed in the Coast Guard regulations on type approval of ballast water management systems (BWMS). The present letter describes DHI's experience with the MPN method for type approval testing of ultraviolet (UV) based BWMS.

DHI is an independent, international consulting and research organisation established in Denmark and today represented in all regions of the world with a total of more than 1,000 employees. DHI Denmark was appointed as a sub-laboratory under the Independent Laboratory (IL) headed by DNV GL by letter or acceptance from U.S. Coast Guard dated 11 June 2013. DHI has no involvement, intellectual or financial, in the mechanics, design or marketing of the products and technologies that are being evaluated.

DHI is conducting biological efficacy performance evaluation tests of BWMS in accordance with the following rules, standards and guidelines:

1. IMO. International Convention for the Control and Management of Ships' Ballast Water and Sediments
2. IMO. Guidelines for Approval of Ballast Water Management Systems (G8). Resolution MEPC.174(58)
3. IMO. Procedure for Approval of Ballast Water Management Systems that Make Use of Active Substances (G9). Resolution MEPC.169(57)
4. U.S. Coast Guard. Standards for Living Organisms in Ships' Ballast Water Discharged in U.S. Waters
5. U.S. Environmental Protection Agency, Environmental Technology Verification Program. Generic Protocol for the Verification of Ballast Water Treatment Technology

DHI has since 2010 conducted biological performance evaluation of UV-based BWMS applying either low or medium pressure UV. Through the practical technology verification, nine different UV-based technologies have been evaluated, and DHI has thus obtained extensive experience with biological analyses of viable organisms after UV treatment.

The Coast Guard regulation defines a ballast water management system as any system which processes ballast water to kill, render harmless, or remove organisms" (33 CFR § 151.1504). The objective of the regulation is to prevent the spread of invasive species when introduced with ballast

water to the receiving aquatic environment. This objective can be achieved by killing the organisms or by destroying their ability to reproduce.

Type approval testing of UV-based BWMS. Ultra violet (UV) radiation is particularly effective against bacteria and smaller organisms in the 10-50 µm size class defined by the regulation. Practical experience from biological performance evaluations conducted by DHI indicates that UV-based BWMS are capable of eliminating live organisms in ballast water by filtration of the larger organisms (≥50 µm) and destroying the reproductive ability of the smaller organisms (10-50 µm). The effect of the ability of the organisms to reproduce may be explained by damage of DNA and RNA caused by the UV radiation, which prevents cell proliferation.

DHI has documented the treatment efficacy of UV-based BWMS by using a MPN assay to verify compliance with the ballast water discharge standard for the 10-50 µm size class. The MPN assay is based on serial dilution of the water sample, and the most probable numbers of the inlet water, control discharge water and treated discharge water are determined by measuring of the fluorescence after two weeks of incubation. This means that the MPN method is directly linked to algal growth and, thus, it is indicative of the ability of the organisms to establish and reproduce in the environment.

The MPN assay only detects the algae that are able to grow under the applied conditions (e.g., natural and added nutrients, light and temperature). Since 2010, DHI has identified algal taxa and species present in the MPN test tubes after ended incubation of inlet and control discharge samples. During a large number of tests, the confirmed growth under the conditions in the MPN assay in percent of the total algal taxa and species observed in the samples has been 72-82% for fresh water, 78-100% for brackish water, and 82-93% for marine water. Furthermore, the most abundant taxa and species in the inlet waters were also among the taxa and species that grew in the MPN assay.

DHI considers the MPN assay as the best available method to determine viable algae in the ≥10 and ≤50 µm size class after UV treatment for the following reasons:

- The MPN assay is objective, readily applicable and can easily be performed on a routine basis with few requirements to equipment
- The MPN assay is sensitive and has been used to identify BWMS performance claims in relation to e.g. UV-transmittance
- Growth has consistently been obtained in inlet samples and control discharge samples, and DHI considers verified growth in the MPN assay with these samples as a specific method performance criterion
- Historical data show that a versatile range of algal taxa and species is capable of growing under the applied conditions in the MPN assay. DHI considers this documentation adequate for having confidence in the MPN assay as a robust analytical method, which can be used to quantify viable algae in source water and ballast water obtained from different geographic regions.

Finally, it should be mentioned that the use of microscopy after staining with CMFDA and FDA, which is prescribed by the ETV protocol, has intrinsic problems that result in false positive counts of organisms treated by UV-based BWMS. Our experience is that CMFDA and FDA fluoresce for several days after UV-treatment in algal cells that are unable to reproduce. Consequently, the total number of organisms ≥10 and <50 µm that are stained with CMFDA/FDA and appear viable markedly exceeds the number of organisms in the same size class that are capable of reproduction as measured by the evaluation of growth.

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