Frequently Asked Questions (FAQs)

What is an invasive species?

The United States Federal Aquatic Nuisance Species Task Force – 2012 states: “Invasive species are any species or other viable biological material (including its seeds, eggs, spores) that is transported into an ecosystem beyond its historic range, either intentionally or accidentally, and reproduces and spreads rapidly into new locations, causing economic or environmental harm or harm to human health.” In other words, according to the United States Federal Aquatic Nuisance Species Task Force – 2012, to be considered an “invasive species”, organisms must be transported into an ecosystem beyond their historic range, AND capable of:

1. reproducing,
2. spreading, and
3. causing harm.

Can non-reproductive organisms cause an invasion?

No, organisms’ ability to reproduce and spread are key elements of invasive species. If the introduced species cannot reproduce, it cannot colonize and hence cannot pose a threat to the native species and natural ecosystem. The destruction of non-native organisms’ ability to reproduce renders the organisms harmless to compete and crowd native species or invade their ecosystem. Accordingly, the inability of organisms to reproduce is equivalent to killing the organism’s ability to become “invasive.” For measuring the invasive capabilities of nonindigenous organisms, ballast water treatment that destroys the reproductive capabilities of organisms is equivalent to destroying their ability to spread and invade, and a treatment process that effects this change is as protective as one that kills organisms outright.

How does ultraviolet light (UV) treat ballast water?

UV treats ballast water effectively by destroying the reproductive capabilities of organisms. UV treatment causes damage to the organism's DNA and RNA, which destroys its capacity for reproduction (*Gieskes and Buma 1997; Sinha and Hader 2002*). Organisms that cannot reproduce, cannot be considered "invasive species". As such, UV provides an effective means of treating ballast water for the purposes of preventing the introduction and spread of aquatic invasive species.

How well known is the MPN method?

The MPN method of estimating the number of viable bacteria in a sample has been used since the early 20th century. The MPN method is used in the U.S. Environmental Protection Agency, the Food and Drug Administration, and other governmental and commercial enterprises as the approved testing methodology to determine the safety of drinking water, milk, and other bacteriologically sensitive media. To determine whether organisms are living, growing and reproducing, scientists take a sample and subdivide (dilute) it by orders of magnitude (frequently 10× or 2×), and assess the presence/absence of growth units (GU) or colony-forming units (CFU) in multiple subdivisions. MPN is a well-known method of obtaining quantitative data on concentrations or numbers of viable organisms from positive/negative (incidence) data.

Does the MPN method measure "living" cells?

In the scientific context, living things are those that exhibit some or all of the following characteristics: homeostasis, cellular organization, metabolism, growth, adaptation, response to stimuli, and reproduction (*Mader, 1994*). Like the ETV Protocol staining (vital stain) method, the MPN method measures one of these characteristics. The MPN method measures the ability for an organism to reproduce; the vital stain method measures the ability for an organism to metabolize. The USEPA Science Advisory Board's report from July 2011 defines a living organism as follows: A viable or living organism is defined as "an organism that has the ability to pass genetic material on to the next generation." (*Efficacy of*

Ballast Water Treatment Systems: a Report by the EPA Science Advisory Board, July 12, 2011, p.75.) As the MPN method is measuring the ability for an organism to reproduce, it is providing a measure of "living" cells by this EPA definition.

Does the vital stain method measure "living" cells?

The vital stain method does not assess the live/dead status of organisms; it evaluates the presence of esterases within organisms and the integrity of membranes which is a measure of the organism's metabolism and structure. In the scientific context (*Mader, 1994*), living things are those that exhibit some or all of the following characteristics:

- Homeostasis
- Cellular organization
- Metabolism
- Growth
- Adaptation
- Response to stimuli
- Reproduction

Will all organism species grow in the MPN method?

Significant research has been conducted to explore the answer to this question including a comprehensive literature search on work to date regarding the matter (*Cullen and MacIntyre, 2015*; *Throndsen, 1978*; *Kaeberlein, et al., 2002*). All evidence finds no basis to support the claim that certain species would have an inherent problem growing out in the MPN method. Experiments conducted at independent test facilities explored organism growth percentages as part of the MPN method development process and found that growth percentages in samples were very high. After identifying optimum growth conditions, growth success conservatively ranged from 96 – 100% on an abundance basis. It is just plain inaccurate to claim that organisms don't grow-out as all evidence, including experiments, has shown that organism growth percentages are very high.

Will all live organisms stain?

No. The vital stain method is blind to many living organisms, and many species do not make stains fluoresce when alive. Examples of this are dinoflagellates and cysts. This is recognized in the ETV Protocol, which states " … cysts in ships' ballast water represent robust ecological hazards … because of their low metabolic state and relative impermeability to stains, it may be difficult to assess [their] viability … The FDA/CMFDA method has yielded variable results with dinoflagellates and cyst-like objects." (*Generic Protocol for the Verification of Ballast Water Treatment Technology, 2010*) In addition, **recent work** has shown that for many species of phytoplankton the fluorescence of stained living cells is not measurably higher than stained killed cells, so live and dead cells cannot be distinguished.

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Is the MPN method as accurate as the vital stain method?

The MPN method is as accurate, if not more accurate, than the vital stain method. There is a common misconception that the vital stain method is highly accurate, based on the results of one incomplete study (*Steinberg et al., 2011*). The study reported that false negative rates were very low for the four sites investigated. Motility was used to judge whether the vital stain method was accurate; however, there are many species and stages of phytoplankton that are non-motile, leading to the high potential of false negatives in the study. This, coupled with many organisms not capable of staining and the lack of difference in fluorescence between live and dead cells, leads to the vital stain method being highly error-prone. The MPN method is certainly just as accurate, and likely even more so, than the vital stain method.

Is the MPN method reliable and repeatable?

The MPN method is highly reliable and repeatable. The assessment of growth/no growth in the MPN method is very objective. It is defined by a fluorescence threshold, measured by a lab grade instrument that can be calibrated to ensure results are repeatable and reliable. Using calibrated equipment, two labs performing the same assay, should get the same result.

Is the vital stain method reliable and repeatable?

Recent work has shown that the vital stain method is not completely reliable. For many species of phytoplankton, the fluorescence of stained living cells is not measurably higher than stained killed cells, so live and dead cells cannot be distinguished. Staining intensities can be very different between species, making the subjective evaluations of mixed assemblages that are required for ballast water samples highly error-prone and difficult to repeat due to the high dependence of the results on the microscopist performing the assay. There is no standard and no control for the value at which the microscopist decides to set the fluorescence threshold for classifying organisms as “live” or “dead”. This can lead to differences in results between samples, between microscopists, and more alarmingly, between test facilities.

Furthermore, the vital stain method does not take into account potential organism repair mechanisms which further negatively impacts its reliability.

A summary presentation of the recent work on vital stain’s lack of reliability and repeatability can be found **here**, and the corresponding full scientific paper recently accepted by the *Journal of Phycology* **here**.

Is the MPN method just as protective of the environment as the vital stain method?

Yes. The MPN method can determine that an organism is not capable of reproduction with the same or higher level of accuracy as the vital stain method (which determines an organism's esterase activity or membrane integrity). The inability of an organism to reproduce is equally protective as "death" in the context of protecting against invasions.

Is the MPN method used in any other industries?

Yes. In many water treatment industries, the MPN method or equivalent grow-out methods are used as a standard practice to assess performance for any treatment technology. Wastewater in the United States is regulated using fecal coliform or total coliform or *E. coli* standards, which are measured using grow-out methods. (*EPA Guidelines Establishing Test Procedures for the Analysis of Pollutants; Analytical Methods for Biological Pollutants in Wastewater and Sewage Sludge: Final Rule, 72 FR 14220, 14225 TABLE IA*) For drinking water, MPN or equivalent grow-out methods are used as a standard practice to assess performance for any treatment technology (*40 CFR 141.74*). In addition, UV treatment systems undergo validation testing for use in drinking water using live microbes and performance is based on grow-out based methods such as MPN (*40 CFR 141.720(d)(2)(ii)*).**Interestingly, the USCG Final Rule allows the use of water from a public water system in the United States as an alternative to installing and operating a ballast water management system (*33 CFR 151.1510*). This public water system has used MPN or equivalent grow-out methods to determine its' compliance.** Also, the MPN method is even used in the Final Rule itself, for the <10 micron size class. All of the bacteria (*E. coli*, *Enterococci*, *Vibrio cholerae*, HPCs) in the discharge standard and ETV Protocol for biological efficacy testing are evaluated using grow-out methods, including MPN.

Can organisms repair themselves after ballast water treatment?

The MPN method takes into account the potential for organisms to "repair" themselves independent of treatment method. During the grow-out process of the MPN method, organisms are provided abundant amounts of energy (light and nutrients). These are the best possible conditions for repair to occur. Accordingly, if an organism is capable of "repairing" itself, then it would do so during the MPN method and be counted in the test results. At the end of the grow-out period, the MPN method assesses the presence of growth units or colony-forming units, *i.e.*, organisms that have the ability to reproduce. If an organism had repaired its ability to reproduce, this result is captured in the MPN method results.

Noteworthy, is that the vital stain method does not include in its protocol any similar assurance. The vital stain method does not take into account the potential for organisms to repair themselves, placing the environment at risk. The MPN method does not have this shortcoming.

What is the impact on the design of a UV-based ballast water management system that uses vital stains versus MPN as the method to measure treatment efficacy?

When compared similarly, a UV-based system that uses the vital stain method, versus MPN, requires grossly more electrical power. The amount of electrical power is so much more that the UV-based system becomes impractical to install for a great majority of ships as both additional space and electrical power capacity is very limited on existing ships. In a recent public statement, DNV – a USCG Independent Laboratory – has highlighted this issue:

"Disinfecting water while ensuring acute kill of the organisms (instead of the ability to reproduce) will require a much more conservative dosage, implying higher power consumption (three to five times) by the UV lamps compared to most systems designed today. It may also imply more operational restrictions, such as minimum holding times (in a

BW tank) and UV transmittance (UV-T) limitation for the BWMS."

Even more troubling is that data has been independently collected showing that, on average, 10 times more electrical power is needed using the vital stain method (versus MPN) to measure treatment efficacy. This ultimately necessitates the design of an impractically large UV system.

There are ways in which to reduce this electrical power ratio, as highlighted in DNV's statement, all of which places the shipowner at significant risk and operational restriction. While maintaining the same flow rate, there are two main approaches to reduce this electrical power ratio to values less than 10:

1. Create, find or select an influent organism assemblage that is easier to treat at the time of testing to ensure a successful pass, and/or
2. Modify, find or select higher water quality.

Both of these approaches, from a shipowner's perspective are impractical, as the vessel, in realistic operation, has no control on either the organisms or water quality encountered. This places the shipowner at significant risk and likely leads to vessels being out of compliance.

Therefore, the vital stain method is impractical for UV-based systems, as it requires higher electrical power and imposes significant operational restrictions. This effectively forces shipowners to choose less environmental friendly ballast water management systems, such as electro-chlorination, which increases the risk of unwanted disinfection by-products and accidental chlorine discharges.

Can UV systems be monitored to show compliance?

Yes. Compliance of UV systems on ships can be monitored by ensuring that the systems are being operated in accordance with the manufacturer's requirements. Many other types of environmental compliance monitoring are accomplished this way, such as drinking water

facilities (*40 CFR 141.720(d)(3)(i)*) in the U.S. that utilize sensors and examine operational integrity (e.g., the power status to the UV lamps). Biological indicator monitoring can also be employed with UV systems, e.g. monitoring for *Escherichia coli* (*33 CFR 151.1511.a.3.ii*), to demonstrate compliance.

What are the environmental risks associated with chemical-based ballast water management systems, such as chlorine-based systems?

Chemical-based ballast water management systems, such as electro-chlorination or chlorine-based systems, use chemicals that need to be neutralized prior to discharge. In this neutralization step, prior to discharge, there is always the risk that complete neutralization does not occur. If complete neutralization does not occur, the aquatic ecosystem is placed in danger as these chemicals, such as chlorine, can dramatically harm marine life if discharged into the environment.

Additionally, it is well known that the chemicals used to treat ballast water, such as chlorine, can create unwanted and toxic disinfection by-products. This topic has been studied extensively and it has been concluded that these disinfection by-products have the potential to be harmful to the environment (*more citations here*). Therefore, with chemical-based systems, the very environment that is trying to being protected from aquatic invasive species, is potentially placed into increased levels of danger through the risk of accidental discharge of chlorine or the creation of toxic disinfection by-products.

UV-based ballast water management systems do not employ chemicals and, therefore, do not have the environmental risks that are associated with chemical-based systems.

Cullen, J.J. and H.L. MacIntyre (2015). On the use of the serial dilution culture method to enumerate viable phytoplankton in natural communities of plankton subjected to ballast water treatment. J. Appl. Phycol. DOI 10.1007/s10811-015-0601-x.

Delacroix, S., Vogelsang, C., Tobiesen, A., Liltved H. (2013). Disinfection by-products and ecotoxicity of ballast water after oxidative treatment–results and experiences from seven years of full-scale testing of ballast water management systems.

Gieskes, W.W.C. and A.G.J. Buma (1997). UV damage to plant life in a photobiologically dynamic environment: The case of marine phytoplankton. Plant Ecol. 128(1-2): 16-25.

Gonsior, M. et al (2015). Bromination of Marine Dissolved Organic Matter Following Full Scale Electrochemical Ballast Water Disinfection. Environmental Science & Technology, DOI : 10.1021/acs.est.5b01474.

Helmholtz Zentrum Muenchen – German Research Centre for Environmental Health (2015).

Kaeberlein, T., K. Lewis, and S.S. Epstein (2002). Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science 296: 1127-1129.

Mader, S (1994). Inquiry into Life; 7th edition, Brown (William C.) Co., U.S.

Shah, A.D., Liu, Z., Salhi, E., Höfer, T., Werschkun, B., von Gunten, U. (2015). Formation of disinfection by-products during ballast water treatment with ozone, chlorine, and peracetic acid: influence of water quality parameters.

Sinha, R.P. and D.-P. Hader (2002). UV-induced DNA damage and repair: a review. Photochemical & Photobiological Sciences 1(4): 225-236.

Steinberg, M.K., E.J. Lemieux and L.A. Drake (2011). Determining the viability of marine protists using a combination of vital, fluorescent stains. Mar. Biol. 158: 1431–1437.

Throndsen, J. (1978). The dilution-culture method. Phytoplankton Manual. Sournia, A. Paris, UNESCO: 218-224.

Werschkun, B., Sommer, Y., Banerji, S (2012). Disinfection byproducts in ballast water treatment: An evaluation of regulatory data. Water Research. 46: 4884-4901. Doi:10.1016/j.watres.2012 .05.034.