The Most Probable Number (MPN) Method to Quantify Organisms ≥10 μm and <50 μm: An Update

Lisa A. Drake[1], Scott C. Riley[2], Stephanie H. Robbins-Wamsley[3], Vanessa Molina[4], and Matthew R. First[5]

.

Chemistry Division, Naval Research Laboratory, Washington, DC 23075

[1]Naval Research Laboratory, Key West, FL 33041

[2]Excet, Inc., Springfield, VA 22150

[3] Naval Research Laboratory, Washington, DC 20375

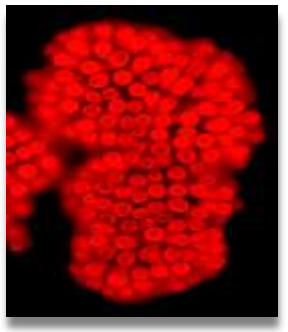
International Conference on Aquatic Invasive Species • 10-14 April 2016 • Winnipeg, Manitoba

Outline

- Determination of ballast water management system (BWMS) treatment effects on organisms ≥10 µm and <50 µm in the context of the current USCG regulation
- Review of effects following treatment by BWMS (e.g., viability)
- Viability (MPN) task group: History and next steps

Quantifying *Any* BWMS's Effects on ≥10 µm <50 µm Organisms

- The ideal method to determine the number of living organisms would:
  - Allow a relatively large volume of water to be sampled
    - Validation (type approval testing)
  - Be relatively quick and straightforward to perform
  - Unambiguously identify all living cells and organisms
- Most traditional metrics are insufficient to *quantify* organisms in a mixed assemblage with confidence: molecular methods, chlorophyll *a*, uptake of radiolabeled substrates (e.g., $H^{14}CO_3^-$)
- The problem (living vs. dead single-celled organisms) is decades old



American Society for Microbiology

Quantifying *Any* BWMS's Effects on ≥10 µm <50 µm Organisms

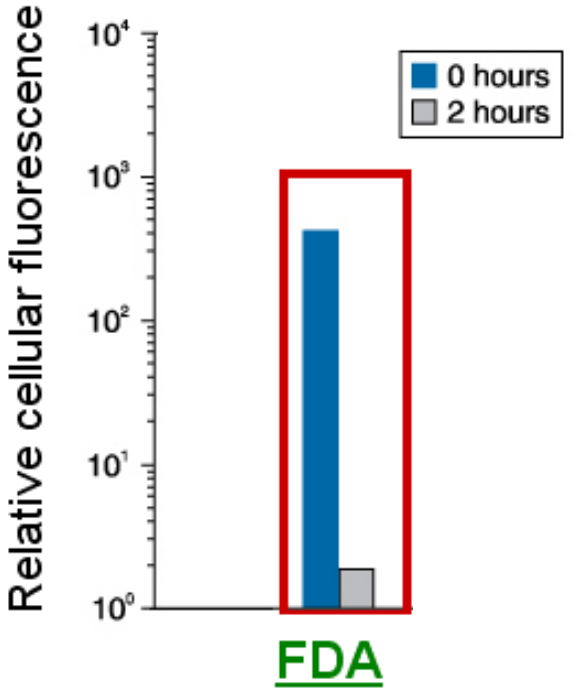
- NRL conducted >100 trials using vital and mortal stains (which indicate if organisms are living or dead, respectively)
- Another option: fluorescent markers that reveal
  - Information about the integrity of enzyme systems
  - Ability of cells to control internal composition

FDA + CMFDA

Fluorescein Diacetate (FDA)

viability probe

Fluorescence decreases over time due to leakage of fluorescein from cells



FDA + CMFDA

Fluorescein Diacetate (FDA)

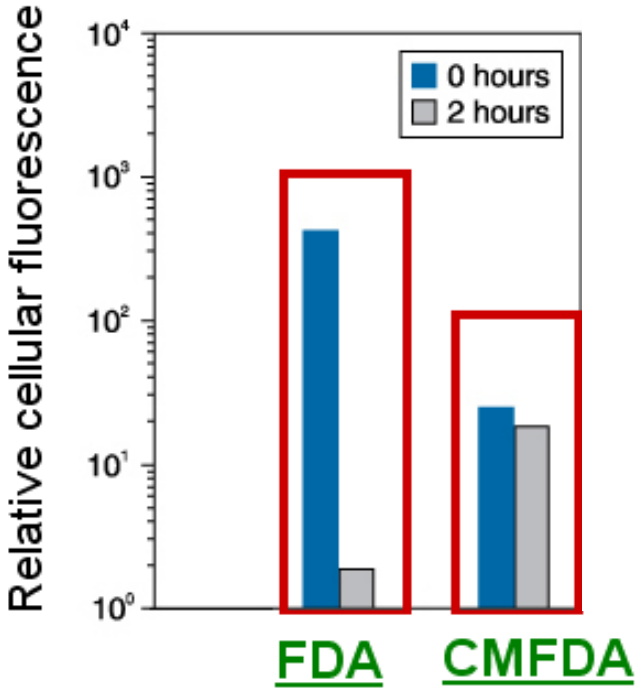
viability probe

Fluorescence decreases over time due to leakage of fluorescein from cells

5-Chloromethylfluorescein Diacetate (CMFDA)

viability probe

Retained inside cells, but the chloromethyl group appears to reduce maximum signal intensity

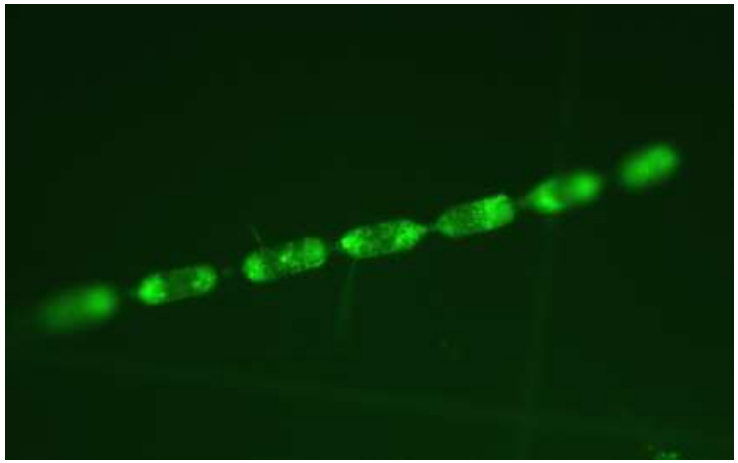


Why not use both?

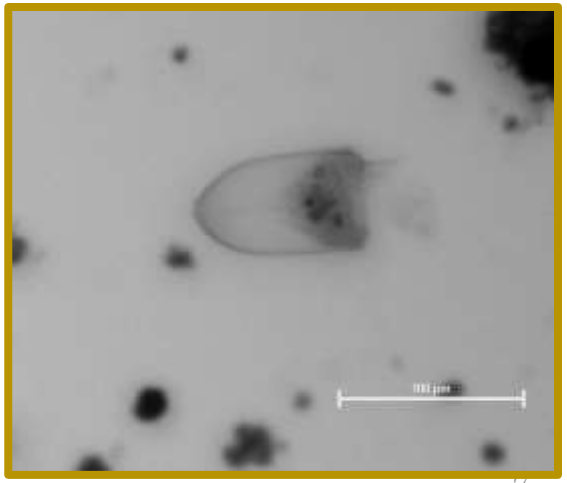
FDA + CMFDA + Movement

FDA/CMFDA + Direct Counting = # Living Organisms

(FDA + CMFDA)



www.microscopy.uk.com



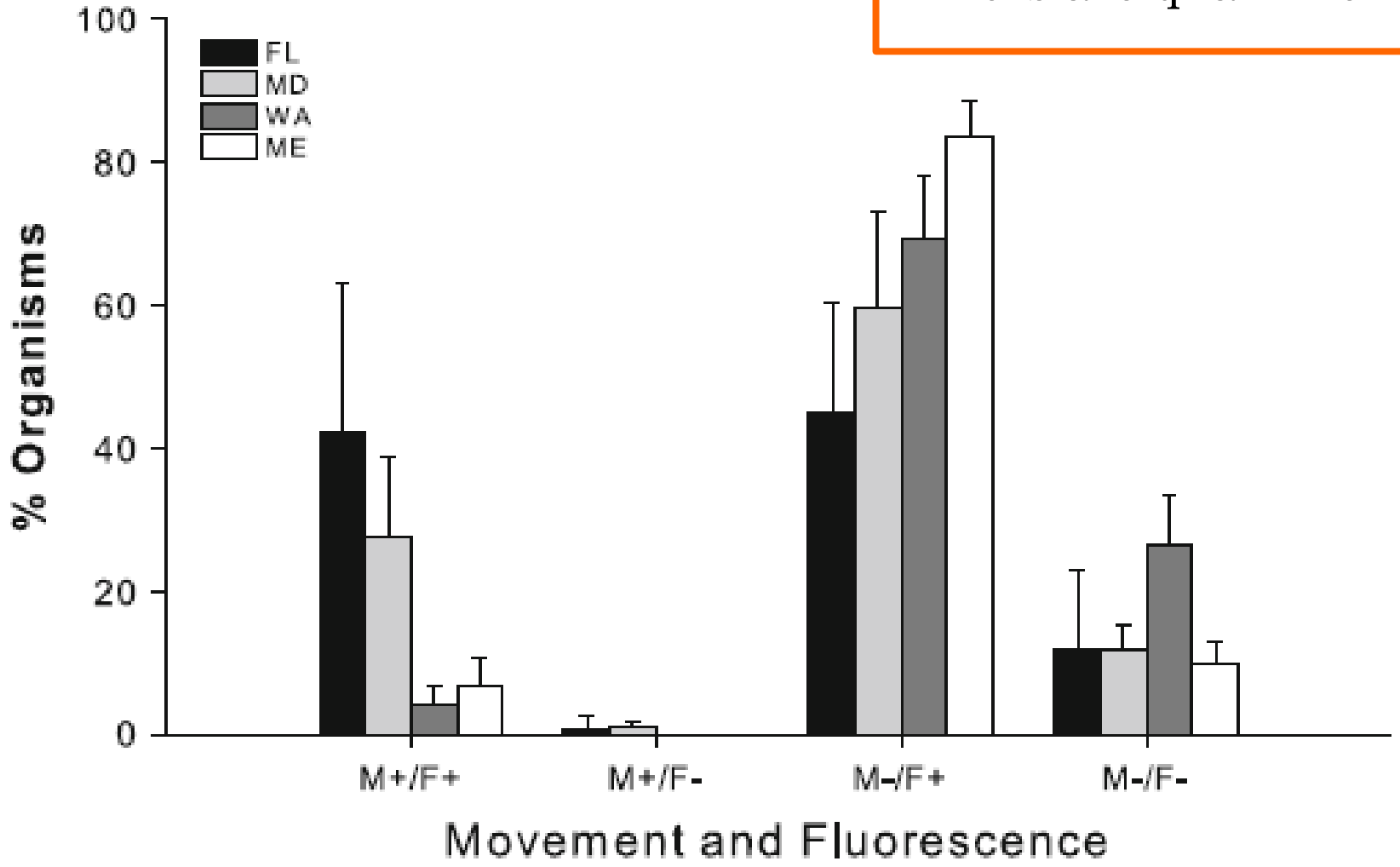
FDA + CMFDA + Movement

- Validation study conducted at four locations with different water types:
  - **Key West, FL**
  - **Baltimore, MD**
  - **Boothbay Harbor, ME**
  - **Sequim, WA**

- Environmental Technology Verification (ETV) Program Technical Panel concurrence
- Peer-reviewed publication (Steinberg et al. 2011)

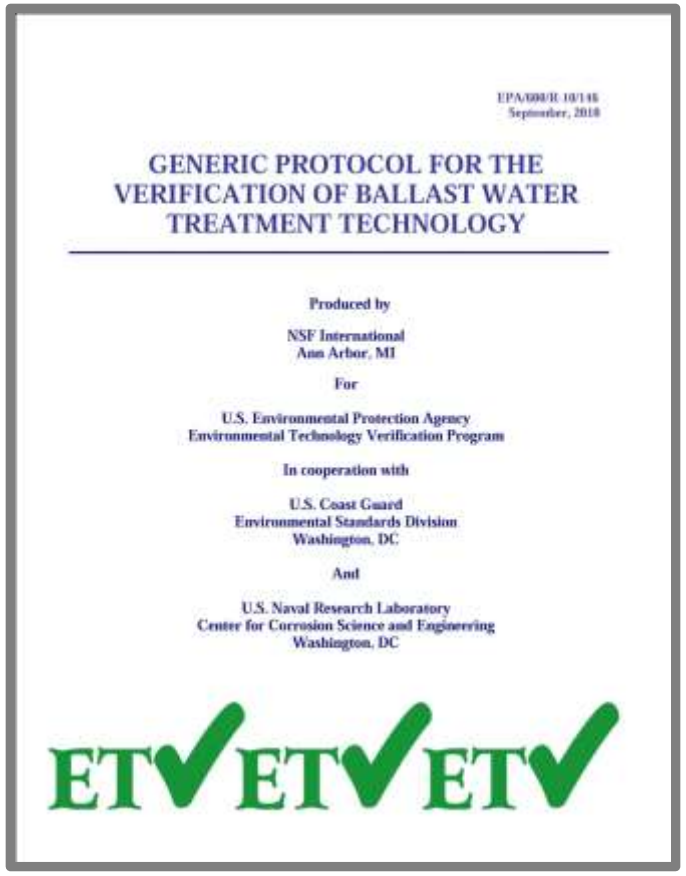
FDA + CMFDA + Movement

Errors are quantified



(Steinberg et al. 2011)

FDA + CMFDA + Movement


EPA/600/R-10/146
September, 2010

GENERIC PROTOCOL FOR THE VERIFICATION OF BALLAST WATER TREATMENT TECHNOLOGY

Produced by

NSF International
Ann Arbor, MI

For

U.S. Environmental Protection Agency
Environmental Technology Verification Program

In cooperation with

U.S. Coast Guard
Environmental Standards Division
Washington, DC

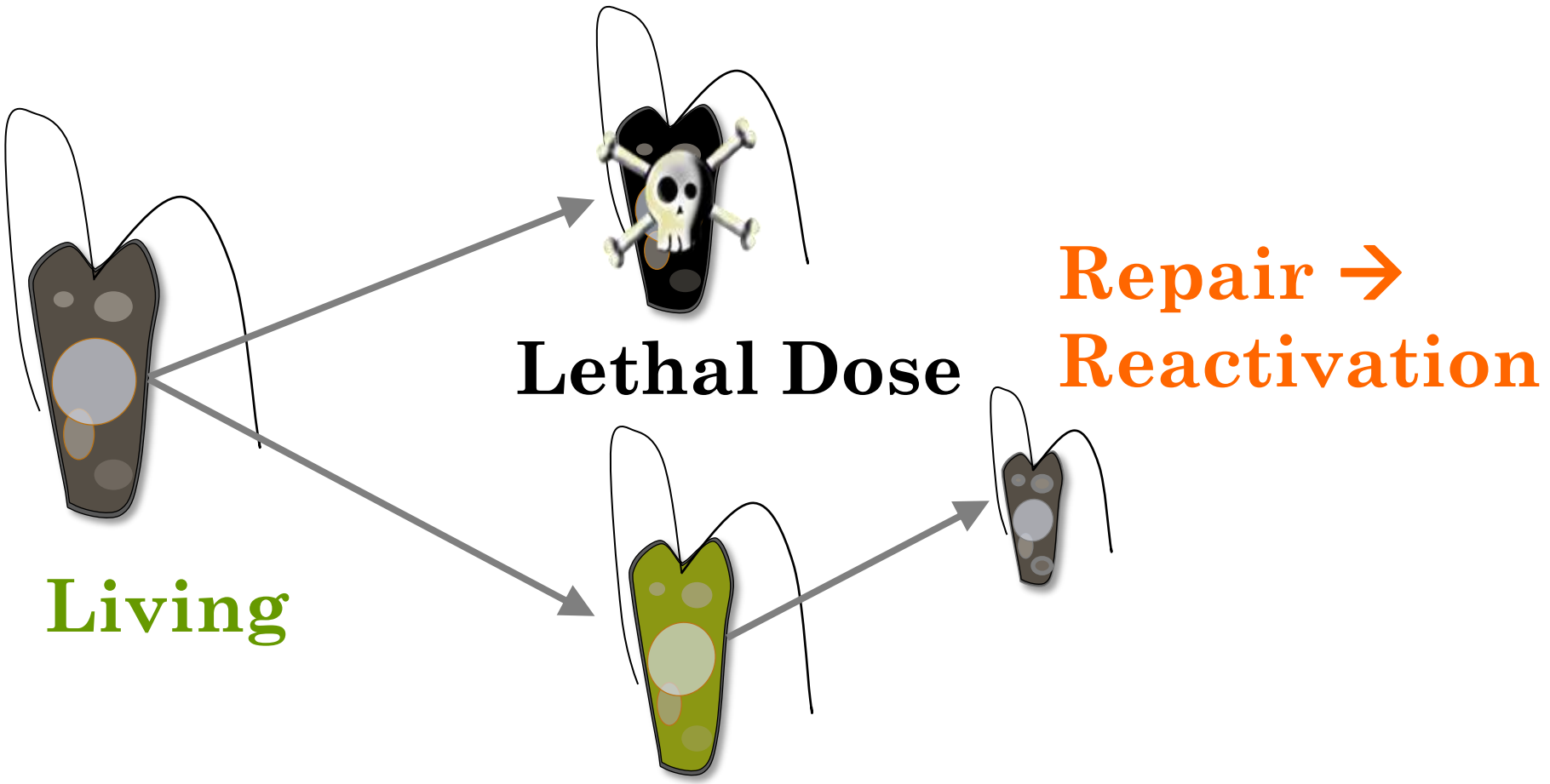
And

U.S. Naval Research Laboratory
Center for Corrosion Science and Engineering
Washington, DC

ETV ETV ETV

- The FDA/CMFDA + movement method is specified in the ETV Protocol
  - USCG Final Rule incorporates ETV Protocol by reference
  - EPA Vessel General Permit currently requires self-monitoring of organisms <10 µm (bacteria and indicator organisms) following sampling consistent with the ETV Protocol
- Alternate approaches may be used
  - Validation would be required

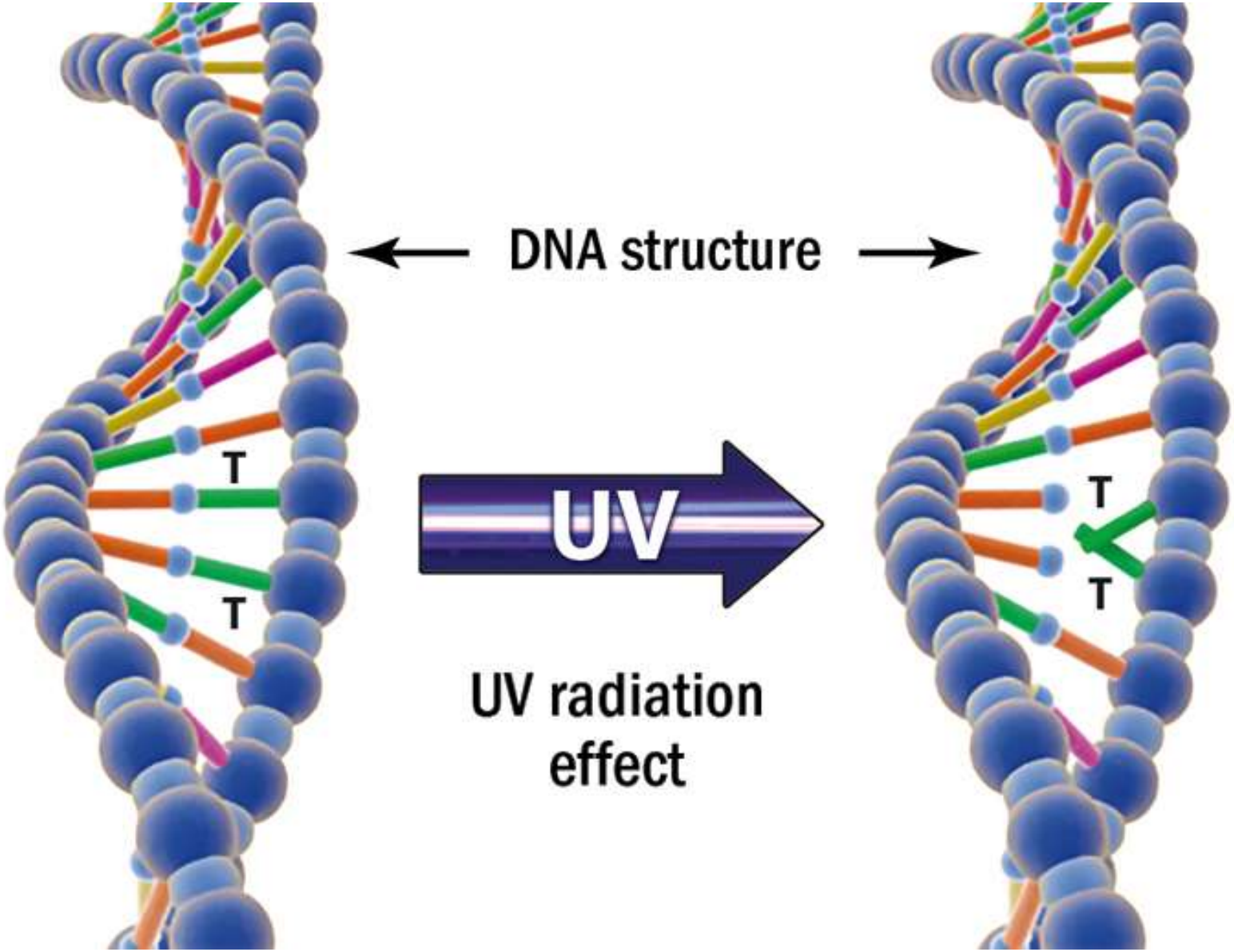
UV-Treated Organisms



Sublethal Disinfecting Dose

Damage: DNA, cell membrane, enzymes

Germicidal Effects

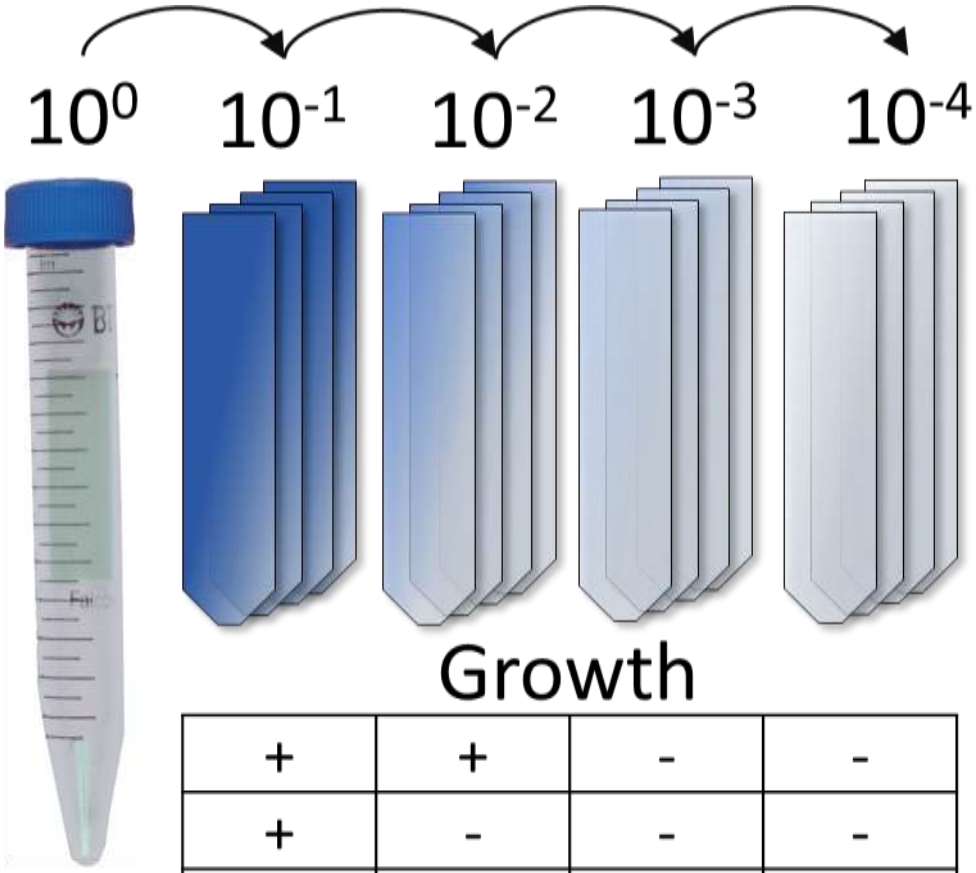


http://www.ultraviolete.com/about-us/

Germicidal Effects

- At doses typically used in disinfection—
- UV covalently links adjacent bases ("rungs" in DNA ladder), causing "**thymine dimers**"
- When the cell divides, DNA replication and transcription is inhibited
- Thus, microorganisms are rendered **unable to divide** and reproduce; death is not immediate

MPN Method



Growth			
+	+	-	-
+	-	-	-
+	-	-	-
+	+	+	-

- Incubation conditions are specified:
  - Temperature
  - Media
  - Illumination
  - Time

MPN Method

- Developed for single-species bacteria cultures
  - Readily culturable under laboratory conditions
- Not all species present in the diverse community of ambient, photoautotrophic protists can be grown in the laboratory
  - The heterotrophs are excluded

MPN Method

- Theory: Dilute cells to the point of absence → calculate the original [ ] (with confidence intervals)
- Top-level scientific questions about the method:
  - How to account for non-photosynthetic (heterotrophic) organisms
    - Proposal: Count with movement + add numbers to MPN results
  - How to determine the percentage of species that can reliably grow during the MPN grow-out period
    - How to account for non-growers (correction factor?)
      - Cultivation issue

MPN Task Group: History

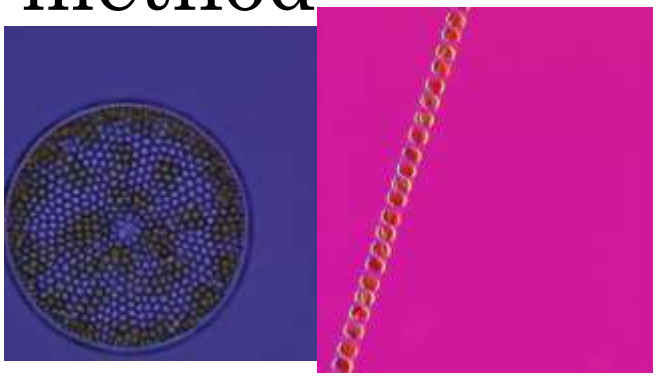
- Initially met in June 2013 through the ETV Technical Panel
- Since that time:
  - 7 teleconferences
  - 2 in-person meetings of full task group

MPN Task Group: History

- Initially met in June 2013 through the ETV Technical Panel
- Since that time:
  - 7 teleconferences
  - 2 in-person meetings of full task group
  - 3 round-robin experiments among three test facilities, preliminary work at DHI Denmark, NIVA, Moss Landing Marine Laboratories
  - 3 additional experiments largely to determine the percentage of species that can grow/be maintained, accuracy, repeatability, etc.
  - 2 in-person meetings of statistics task group

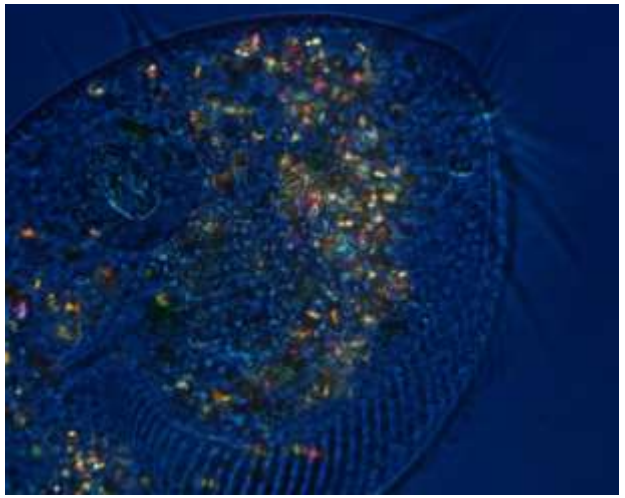
MPN Task Group

- The **MPN method** was drafted, including
  - Summary of data on multiple species analysis from the test facilities
  - Autotroph and Heterotroph method
  - SOP for autotrophs
    - One temperature
    - One media
    - A threshold for determining whether tubes have growth
      - 4x the SD of the fluorescence of a set of blank tubes



MPN Task Group

- Summary of interferences, biases, and limitations
- Heterotroph Method
  - Evaluated by
    - Presence of motility
    - Absence of chlorophyll *a* autofluorescence



MPN Task Group

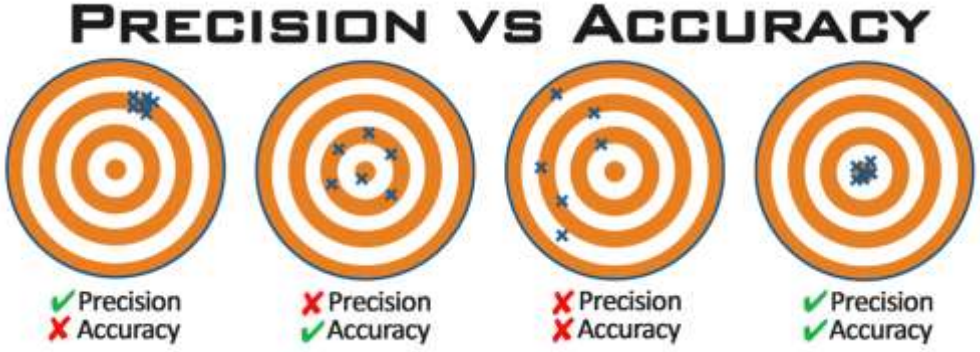
- A step-by-step standard operating procedure has been developed
  - At two facilities, the percentage of non-photosynthetic (heterotrophic) organisms was low, ≤1%
  - The percentage of species that ***can grow consistently*** ranged from **20-44% or 56-89%**
  - The percentage of species that ***grew in at least one test*** ranged from **80-89% and 70-94%**
    - That is, the "historical record" was greater than the percentage of species that consistently grow in each test

MPN Task Group—Statistics

- A **modeling study** is being conducted
  - Determining the effect of non-growing species on the final concentration of living organisms
    - Unaccounted for living organisms represent false negatives
      - How can they be incorporated?

MPN Task Group—Statistics

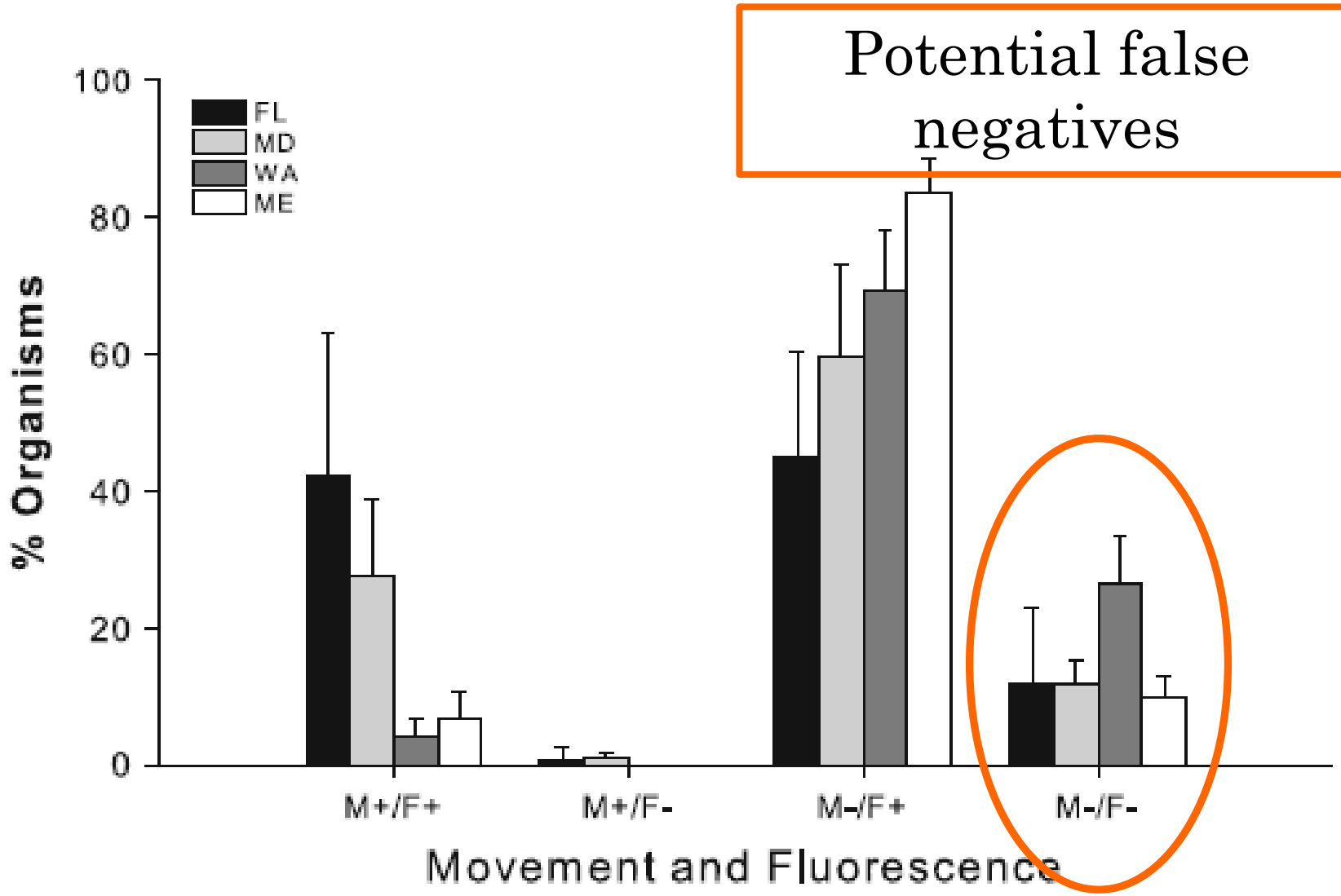
- An **uncertainty analysis** is being conducted
  - Accounting for the sources of error
    - MPN method
    - FDA/CMFDA + movement approach
  - Any errors common to both approaches (e.g., error in sample collection) will not be considered



MPN Task Group

- A **validation experiment** is being planned now and will be conducted, including
  - Known standards (e.g., living cells, heat-killed cells)
  - Documentation of precision, accuracy, etc., for the method

MPN Task Group—Statistics



(Steinberg et al. 2011)

Acknowledgements

USCG Environmental Standards Division
(CG-OES-3)
Richard Everett and Regina Bergner

EPA Office of Research and Development
Ray Frederick

NSF International
Tom Stevens

Naval Surface Warfare Center Carderock Division
Mia Steinberg

NRL
Elizabeth Hogan, Acting Section Head of NRL Code 6136 and Director of the Center for Corrosion Science and Engineering
Key West, FL