Most Probable Number (MPN) Assay to Determine Concentrations of Ambient Organisms ≥10 µM and <50 µM in Oligotrophic Waters

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

Chemistry Division, Naval Research Laboratory, Washington, DC 23075

[1]Excet, Inc., Springfield, VA
[2]Naval Research Laboratory (NRL), Washington, DC
[3] NRL, Key West, FL

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

Introduction

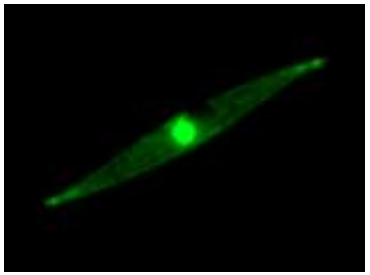
- U.S. Coast Guard (USCG) and Environmental Protection Agency (EPA) set limits for number of living organisms discharged in ballast water to minimize delivery of aquatic nuisance species (ANS) to U.S. waterways

- To meet discharge standards, most ships will install ballast water management systems (BWMS), subject to a regime of verification tests

- BWMS are subject to land-based and shipboard testing verifying efficacy meeting the discharge standard

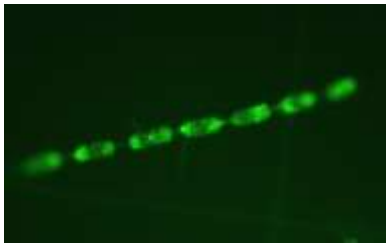
"Required" Method (ETV* Protocol)

- ≥10 µm and <50 µm sized organisms



- Fluorophore labeling:
  - Fluorescein diacetate (FDA)
  - Chloromethylfluorescein diacetate (CMFDA)

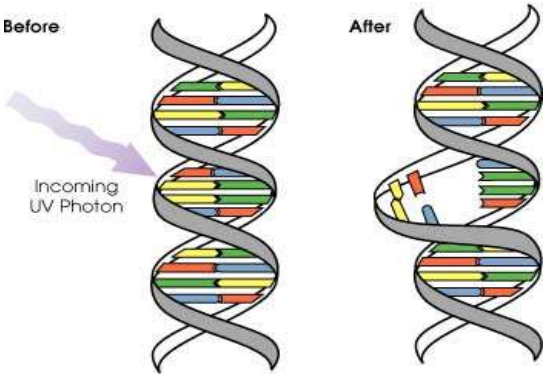
- Fluorescing organisms, and non-fluorescing but moving organisms detected by microscopy and scored as living



*U.S. Environmental Protection Agency, 2010; ETV is U.S. protocol for land-based verification testing of ballast water management systems

Challenge

- Some BWMS employ ultraviolet (UV) radiation
  - Doses commonly used do not necessarily kill organisms immediately but render organisms reproductively sterile



- Required Method
  - No differentiation between organisms capable of *reproduction*, and living, *sterile* organisms (irreparable damage prevents reproduction)

Image credit: http://blogs.scientificamerican.com/observations/subatomic-sunscreen-how-light-particles-can-repair-uv-damaged-dna/

Proposed Alternative Method

- Two approaches to enumerate organisms:
  - **Autotrophic Method**
    - Photoautotrophic reproductive organisms detected by Most Probable Number (MPN) assay
  - **Heterotrophic Method**
    - Heterotrophic organisms detected by epifluorescence microscopy and evaluated for movement and absence of chlorophyll *a* (Chl *a*) signal

Research Goals

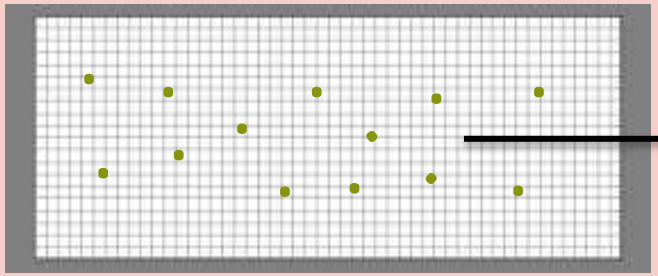
- Compare Alternative and Required Methods using ambient samples from marine, oligotrophic waters

- Measure community composition and size distribution

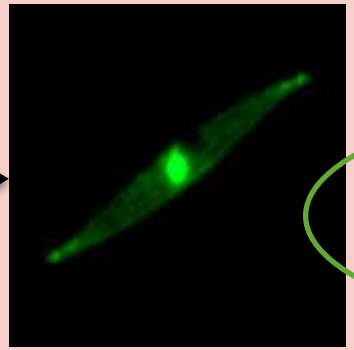
Methods: Required Method

Living organisms

1 mL



Sedgewick-Rafter slide



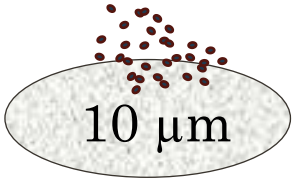
No fluorescence + no movement = **dead cell**

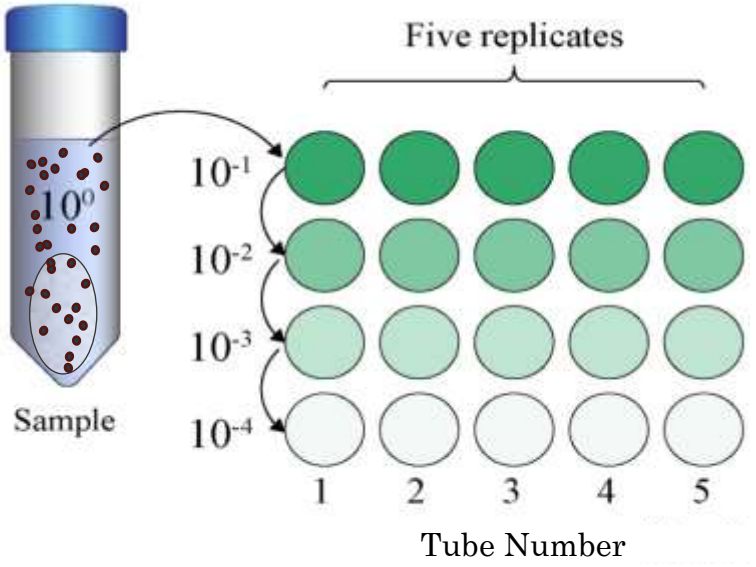
Green fluorescence= **live cell**

Movement = **live cell**

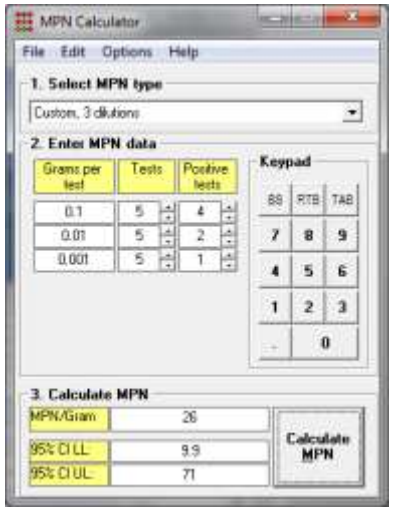
Methods: Alternative Method

Autotrophs





MPN Calculator



Growth scoring pattern

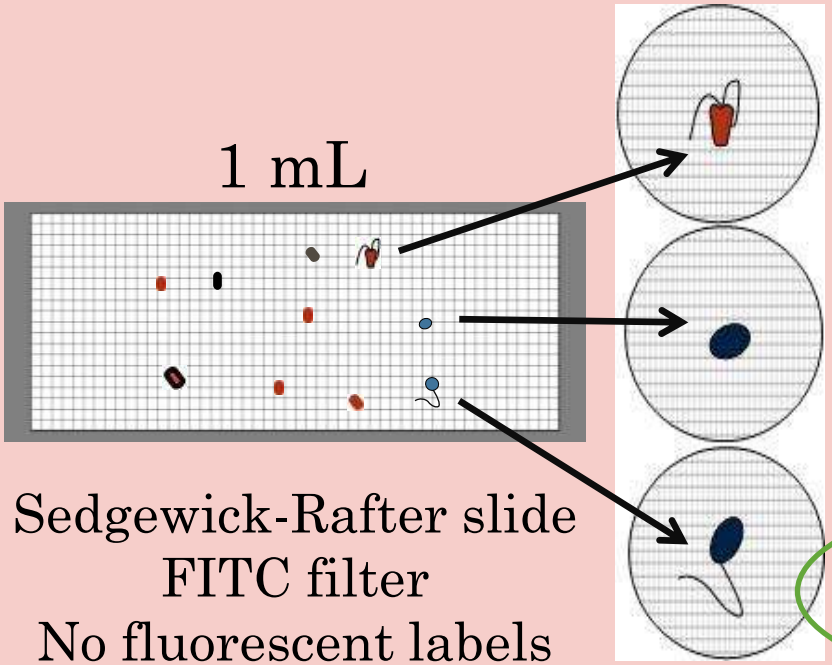
+	+	-
+	-	-
+	-	-
+	+	+
-	-	-

in vivo
chlorophyll *a* readings (µg L^{-1})
Day 0 and Day 14

http://www.hellotrade.com/suppliers?ss=chlorophyll+fluorometer

Methods: Alternative Method

Heterotrophs



Sedgewick-Rafter slide
FITC filter
No fluorescent labels

Red autofluorescence signal = **autotroph**

No red autofluorescence signal + No movement = **dead heterotroph**

No autofluorescence signal + movement = **live heterotroph**

Methods: Alternative Method

- **Autotrophs**: *In vivo* Chl *a* measurements at Day 0 and Day 14
  - Growth measured* by increased Chl *a* fluorescence
  - MPN calculator used to determine concentration of sample
- **Heterotrophs**: Count live organisms ***without*** autofluorescence and ***with*** movement

Total live count = Autotrophs + Heterotrophs

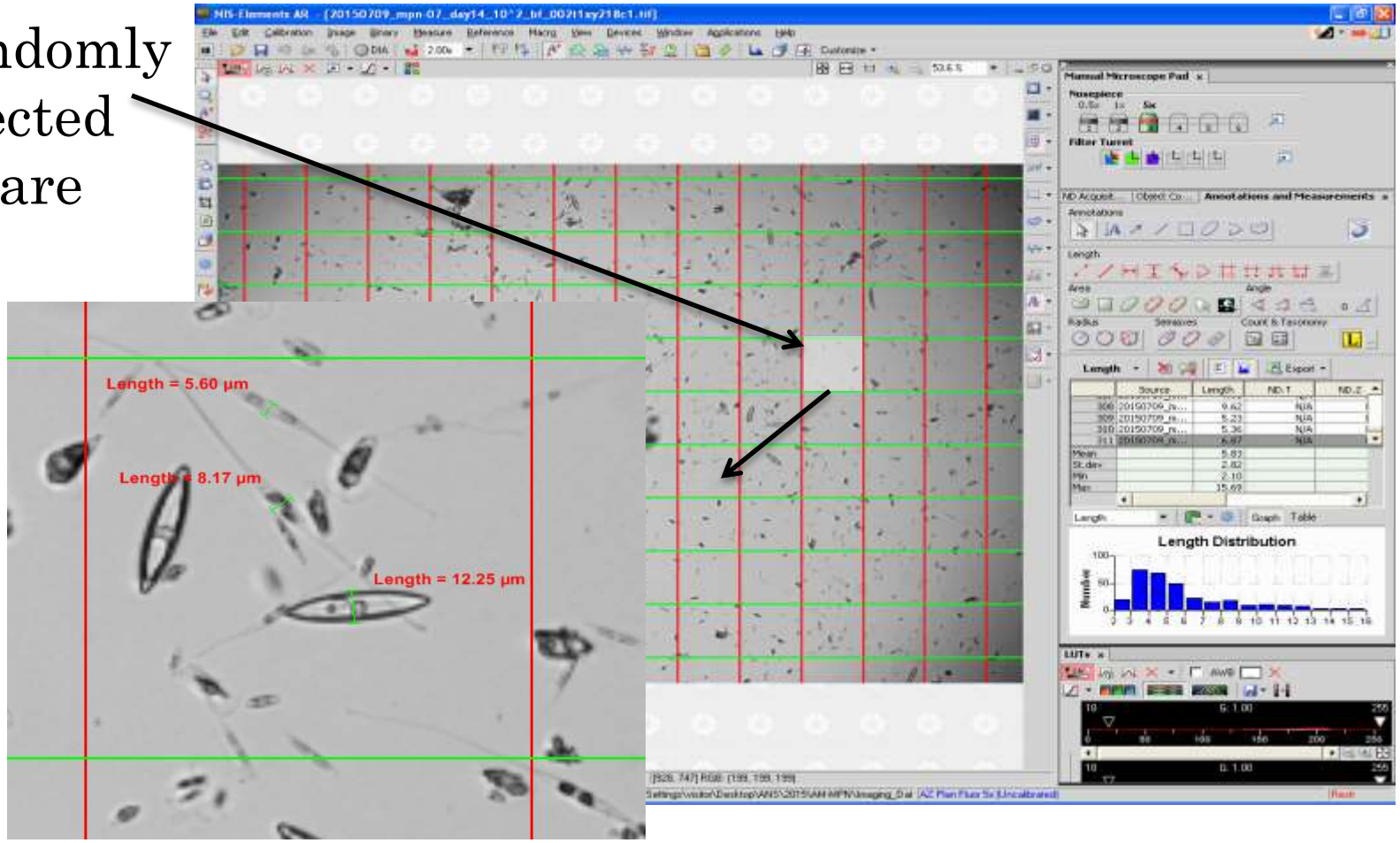
*>4x SD fluorescence of blanks

Methods: Alternative Method Community and Size Distribution

- Automated imaging
  - Detect changes in organism community over incubation period
  - Measure size distribution

Methods: Alternative Method Size Distribution

Randomly selected square



Lengths collected and automatically recorded for each organism in 10^{-2} dilution

Results: Measured Concentrations

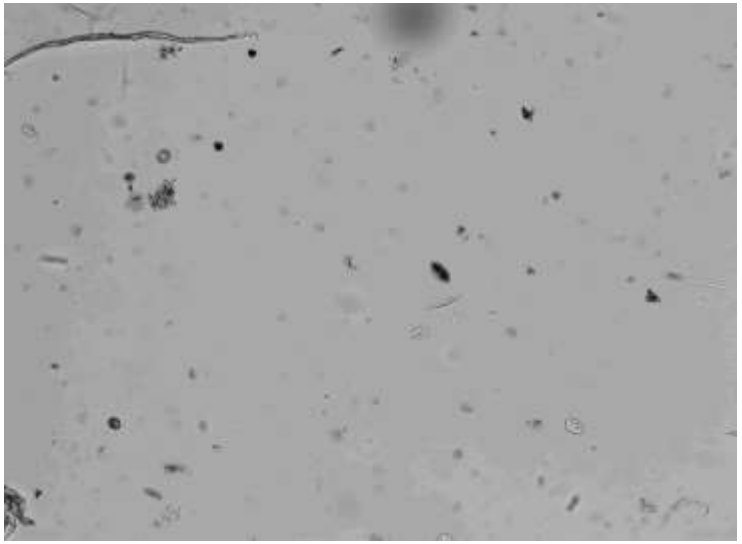
	Alternative Method			Required Method	
	Autotroph Method (mL^{-1})	Heterotroph Method (mL^{-1})	Total ≥10 and <50 µm Organisms* (mL^{-1})	Total ≥10 and <50 µm Organisms (mL^{-1})	CV (%)
Trial 1	>2100	9	>2109	976 ± 43	4
Trial 2	>2100	81	>2181	1201 ± 30	2
Trial 3	>2100	49	>2149	931 ± 128	14
Trial 4	>2100	187	>2287	1656 ± 97	6
Trial 5	2100	220	2320	2193 ± 71	3

*When present, greater symbol (>) was retained from MPN for calculations of total living organisms

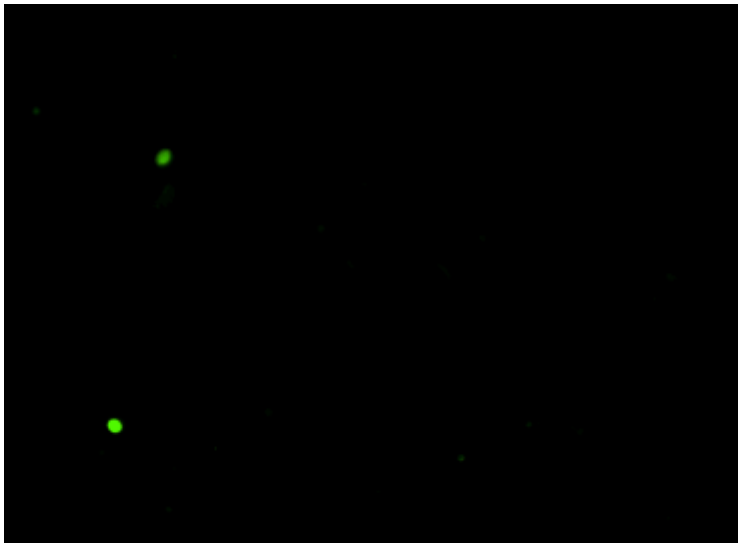
Results: Alternative Method Community Composition

- Automated imaging to detect changes in organism community over incubation period

Concentration Day 0



Total Community

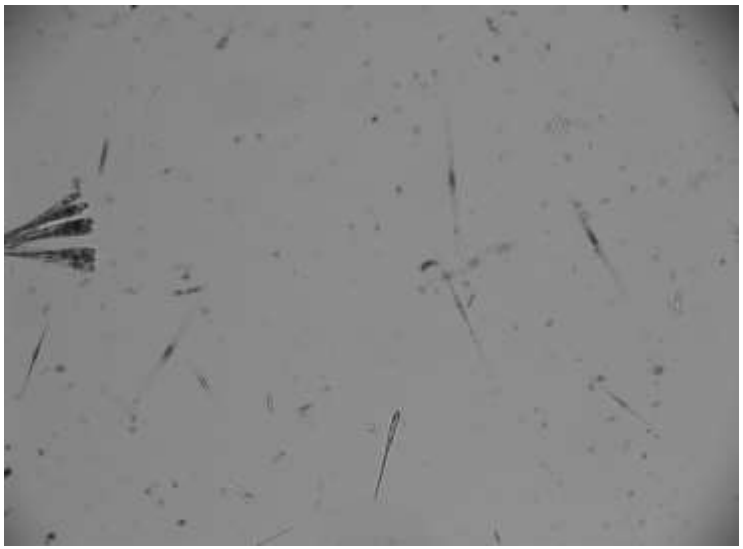


Living organisms

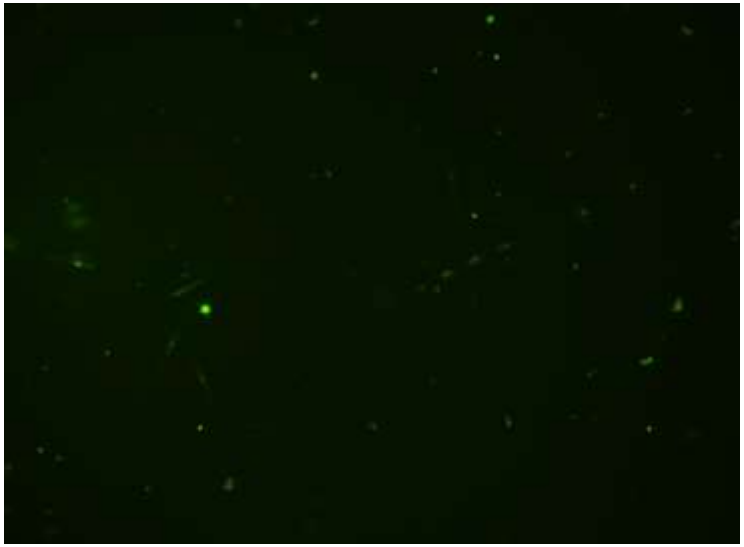
Results: Alternative Method Community Composition

- Community diversity and live organisms following 7 days of incubation

Concentration Day 7



Total Community



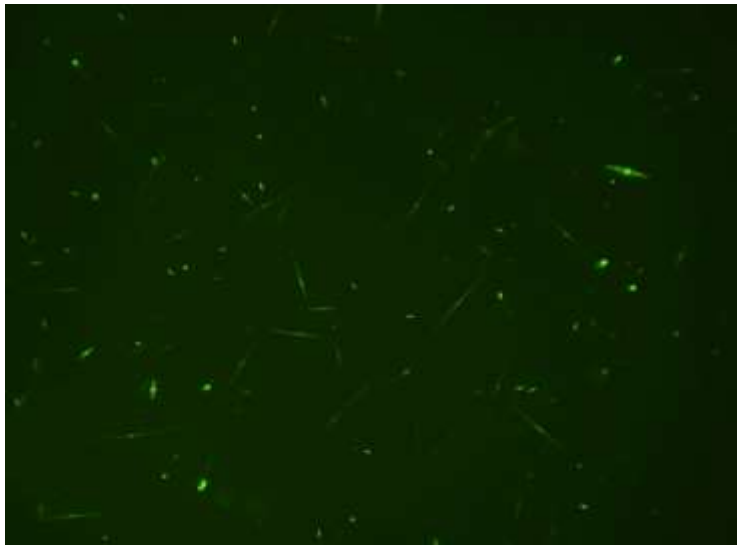
Living organisms

Results: Alternative Method Community Composition

- Community diversity and live organisms following 14 days of incubation

Concentration Day 14

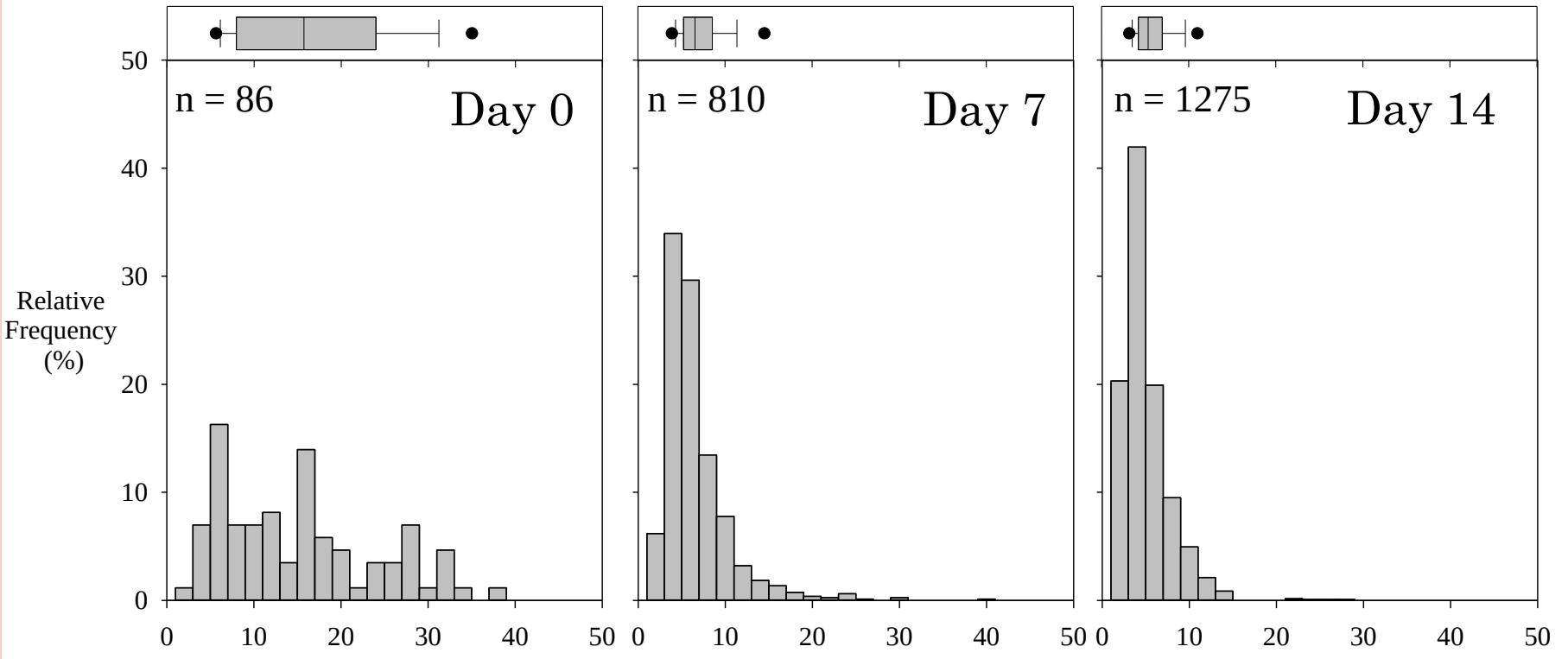
Total Community



Living organisms

Results: Alternative Method Size Distribution

Mean length of organisms found in one dilution (10^{-2})



Day	Mean Length (± 1 SD) (µm)
Day 0	17.1 (±10.6)
Day 7	7.5 (±3.9)
Day 14	6.0 (±2.7)

Conclusions

- In these trials, it was not possible to make accurate comparisons of organism concentrations between Alternative and Required Methods due to inequalities (e.g., >2100 mL^{-1})

- Required Method provides more precise counts than Alternative Method when all tubes result in positive growth

Conclusions

- Using the Alternative Method, organism size distribution indicated diatoms <10 µm in size dominated community at Day 14

- Possible overestimation of organisms ≥10 and <50 µm due to change in fluorescence driven primarily by organisms <10 µm

Acknowledgements

This work does not represent the official position of the USCG Environmental Standards Division (MIPR HSCG23-13-X-MMS010);

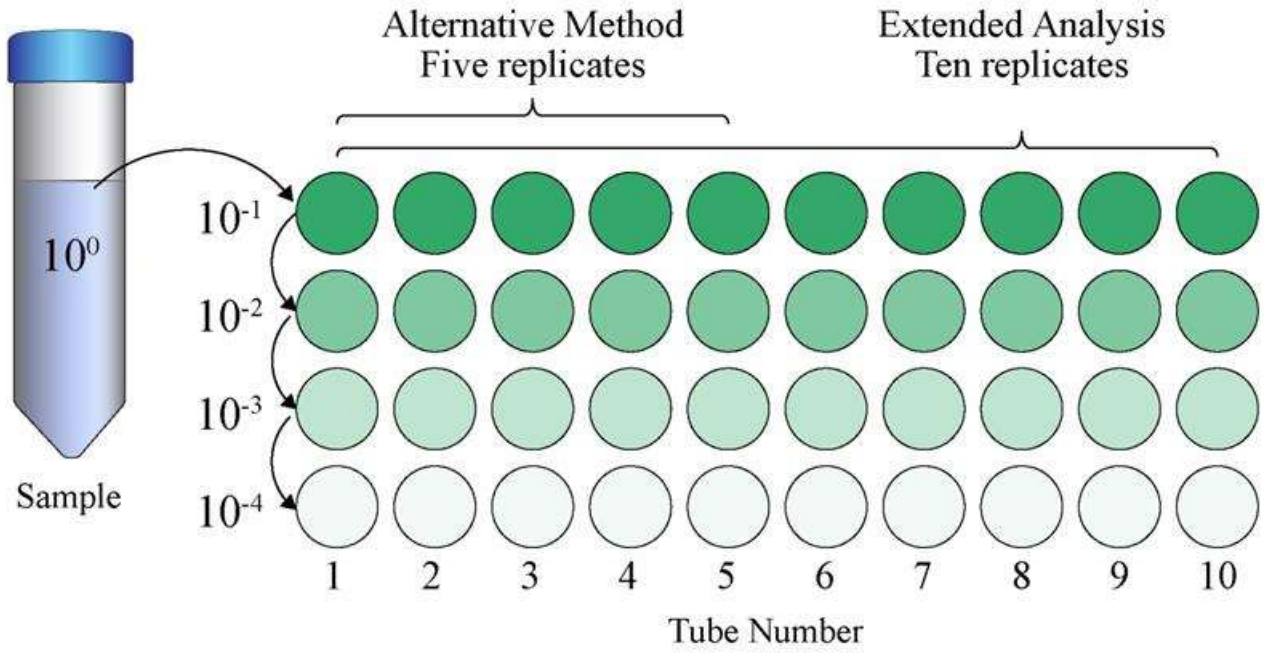
We thank Richard Everett and Regina Bergner for their programmatic support

This work was supported by Elizabeth Hogan, Interim Section Head(Code 6136)
Center for Corrosion Science and Engineering
Key West, Florida

SUPPLEMENTAL SLIDES

Chemistry Division, Naval Research Laboratory, Washington, DC 23075

Methods: Extended Analysis



- Based on 10 replicate tubes for each dilution
  - Compared to MPN analyses with 5 replicate tubes

Results: Extended Analysis

- Positive growth for all dilutions and replicates in 4 out of 5 trials.
- Concentration estimates above MPN calculator threshold (>3000), with lower and upper 95% confidence interval of 1300 and 6600, respectively
- One trial (Trial 5) resulted in 3000 cells mL^{-1} with lower and upper 95% confidence interval of 1300 and 6600, respectively

Conclusions: Extended Analysis

- Additional replicates, in this case, did not substantially change outcome of experiment
  - 4 of 5 trials still resulted in undefined estimate