How effective are size-separation techniques for concentrating live organisms ≥10 µM and <50 µM?

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Introduction: Size-Selective Filtration

- Obtaining accurate measurements of living organism concentrations in ballast water are important for both verification testing and shipboard compliance testing
- Some measurement approaches require a concentration step (e.g. when dealing with sparse populations) or a pre-filtration step (e.g. for increased size-selection of organisms)
- Filtration methods do not result in perfect size fractionation
- Physical filtering process can induce stress, mortality or loss of organisms
- These factors can lead to an underestimation of living organism concentrations

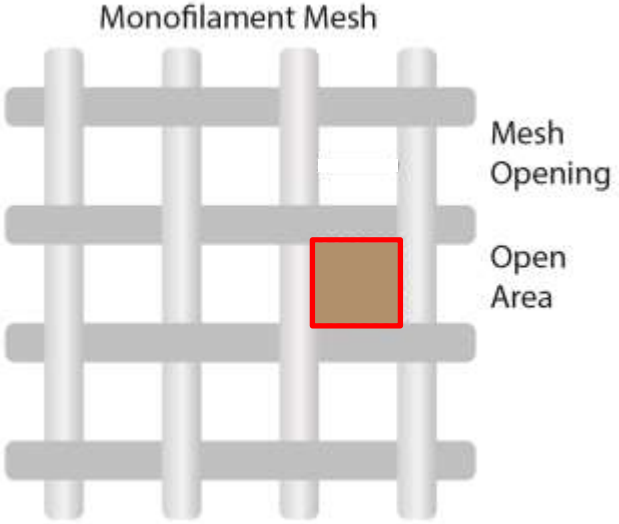
Introduction: Size-Selective Filtration

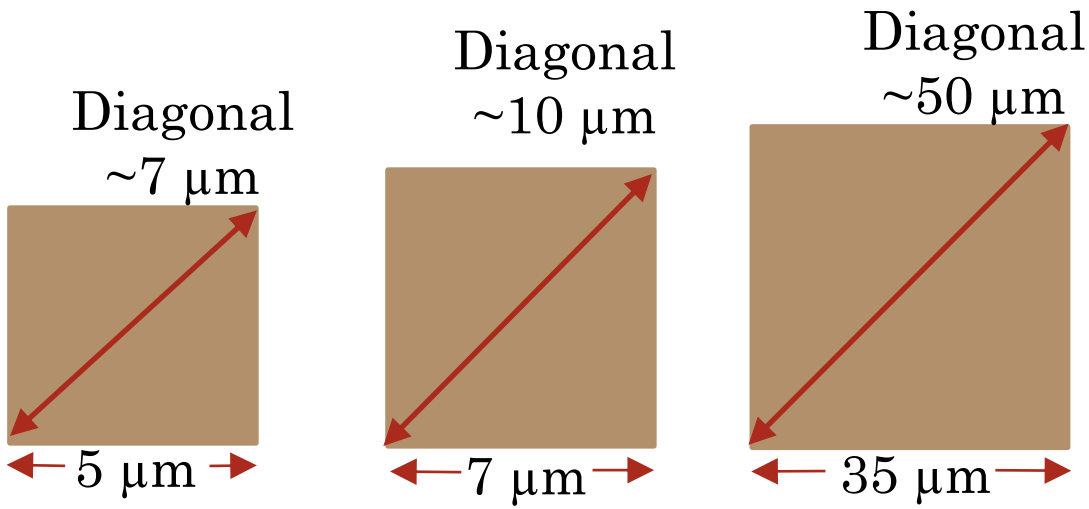
- Examine sparse assemblage concentrations (~10 organisms mL^{-1}) of the ≥10 and <50 µm size class (nominally protists)

- Measure differences in methods of sample filtration used in ballast water testing
  - Ambient marine plankton and laboratory microalgae cultures
  - Retention efficiency (RE)
  - Performance of mesh types and filtration configurations
  - Physiological changes (reduction in fluorescence or increase in mortality)

- Recommend optimal materials and procedures to improve analytical approaches for filtering organisms in the ≥10 and <50 µm size class

Idealized Mesh Types

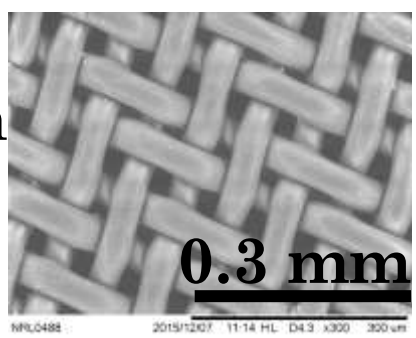


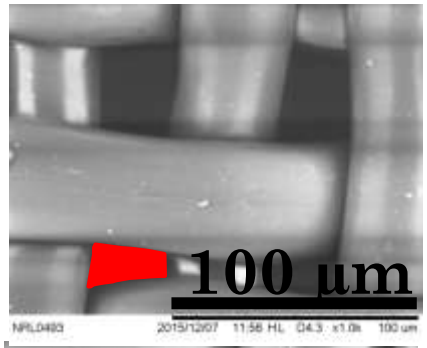


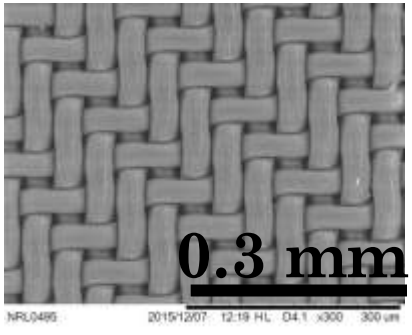
Mesh Type and Nominal Pore Size	Theoretical Size of Organisms Retained (µm)
Nylon (5 µm)	≥7 µm
Stainless steel (5 µm)	≥7 µm
Single Nylon (7 µm)	≥10 µm
Double Nylon (7 µm)	≥10 µm
Dual Nylon(35 µm; 7 µm)	≥10 and <50 µm on 7-µm mesh

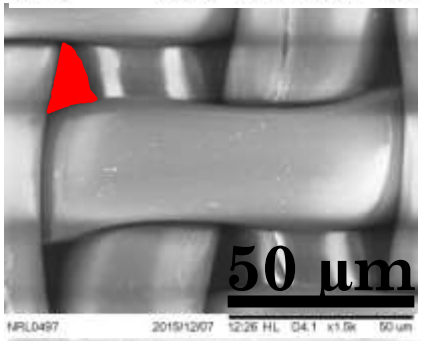
Mesh Types: The Reality (SEM images)

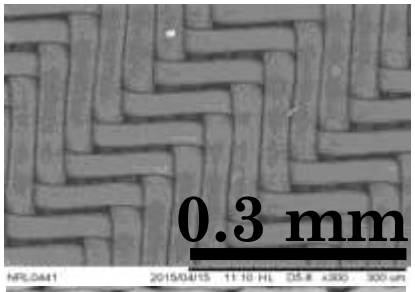
- 35-µm Nylon
- 7-µm Nylon
- 5-µm Nylon
- 5-µm Metal

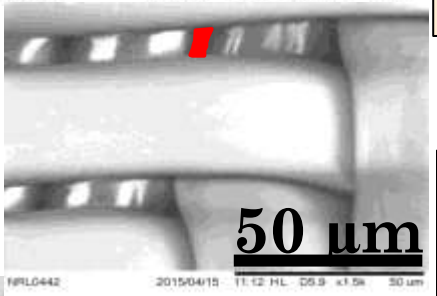


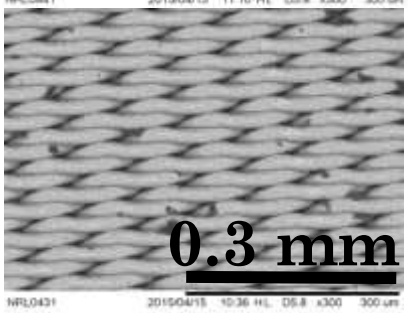


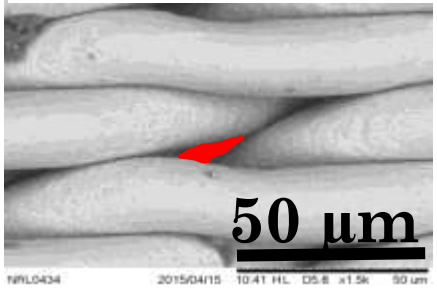










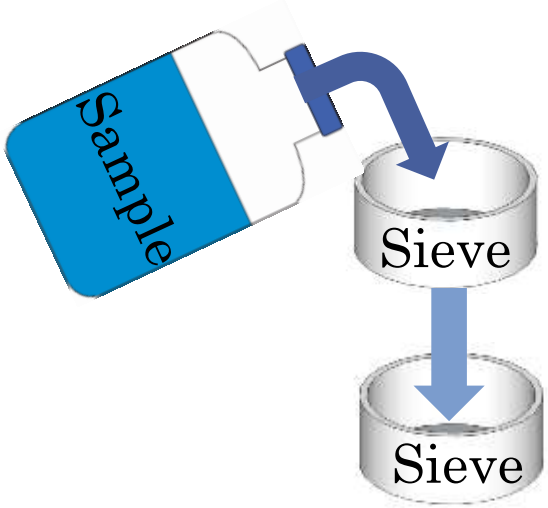


- Mesh weave (3D geometric configuration—not a standard square)
- Accentuated with decrease in nominal pore size
- How will configuration differences effect organism retention and mortality?

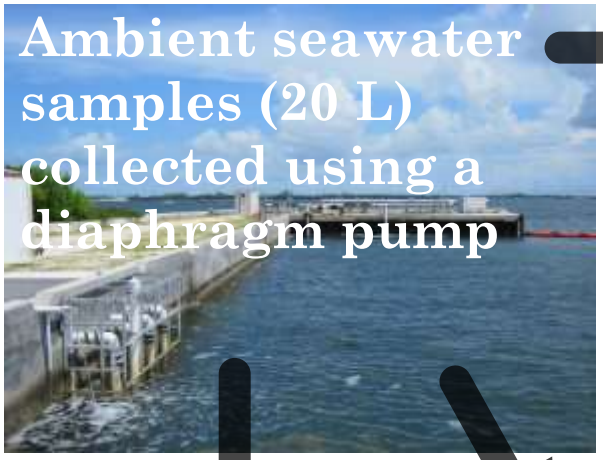
Filtration Approaches

- **No Filtration:** Whole water
  - Used for comparison to filtered samples
- **Single-Stage Filtration:** Sample passed through **one sieve**
  - Used for direct counts via epifluorescence microscopy
  - Organisms ≥50 µm are present but visually excluded from total counts

- **Double Filtration:** Sample passed through a series of **two sieves** with the **same mesh** size (ex. two 7-µm mesh sieves)
- **Dual-Stage Filtration:** Sample passed through **two sieves** with **different mesh** size (here, 35-µm then 7-µm mesh)
  - Minimizes interferences from organisms ≥50 µm



Sample Collection: Mixed Ambient Community

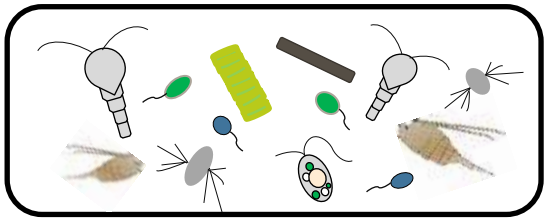


Dual Stage Filtration

20 L

35 µm

Filtrand



20 L → 20 mL (1,000x)

Whole water (No Filtration)

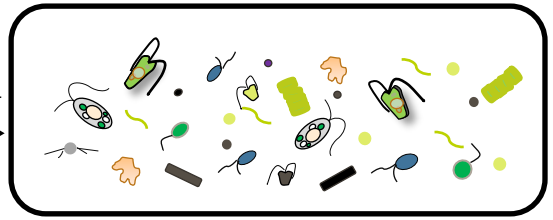
20 L

Single-Stage Filtration

20 L

5 or 7 µm

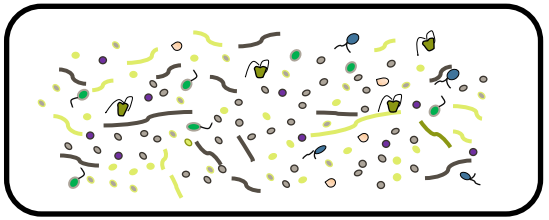
Filtrand



20 L → 75 mL (375x)

0.22 µm

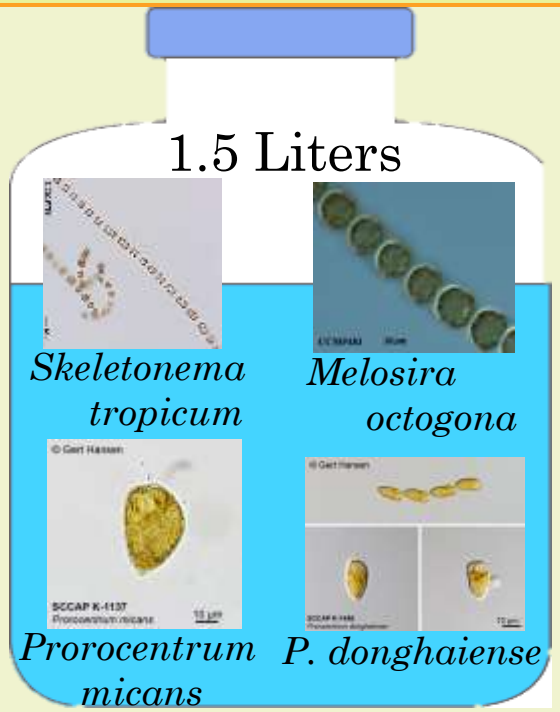
Filtrate



2 L → 75 mL (37.5x)

Sample Collection: Cultured Microalgae

Source Culture Water



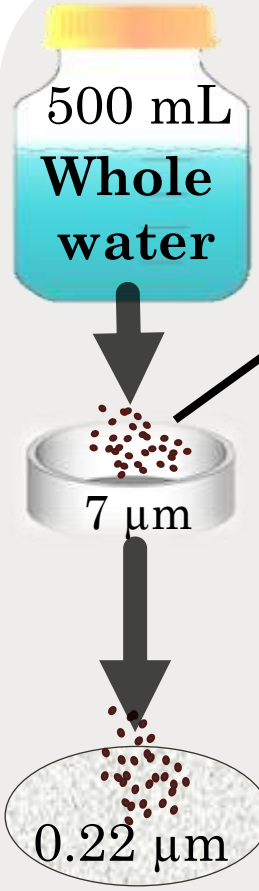
› 4 species per trial
  › 2 chain species
  › 2 non-chain species

› Concentration: 1,000 living organisms mL^{-1}
› (≈ 250 mL^{-1} per species)

No Filtration

Three (3) subsamples

Single-Stage Filtration

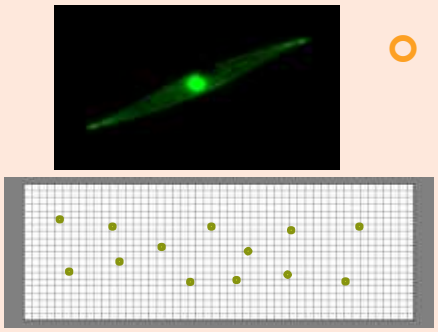


Three (3) subsamples

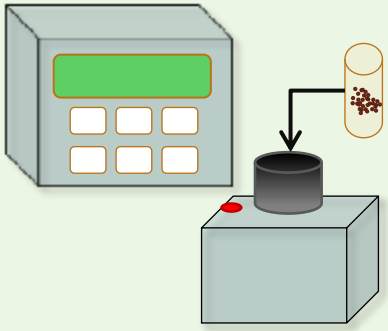
Three (3) subsamples

Sample Analysis: All Sample Types

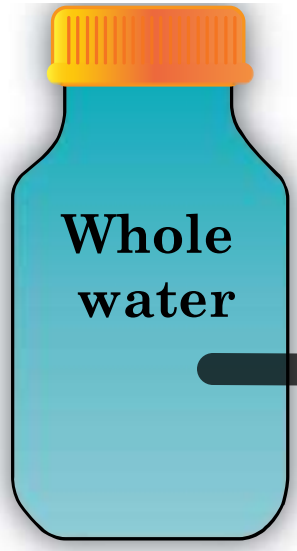
Sample Analysis Suite: 3 subsamples taken for each analysis type

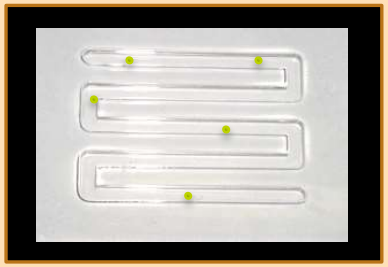


- **Epifluorescent microscope counts**
  - FDA/CMFDA fluorochromes
  - 1-mL volume on Sedgewick-Rafter counting chamber



- **Variable fluorescence**
  - Pulse Amplitude Modulation (PAM) fluorometry
  - 3-mL volume
  - 0 Day sample analyses

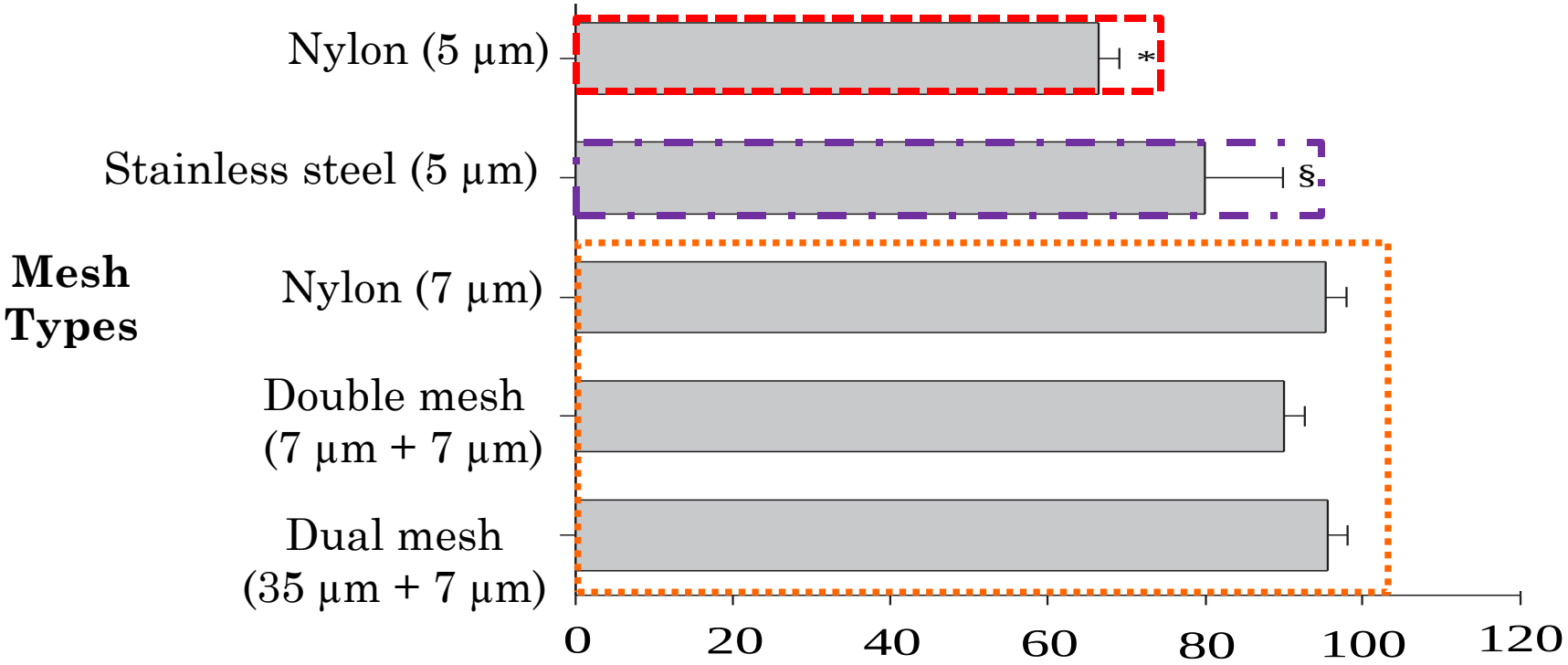




- **Epifluorescent microscope counts**
  - FDA/CMFDA fluorochromes
  - 5-mL volume in a Bogorov counting chamber

Results: Living, Ambient Organism RE

Calculated as: **Filtrand divided by filtrand + filtrate**



Bars = mean, 1 SD
n = 3 to 6

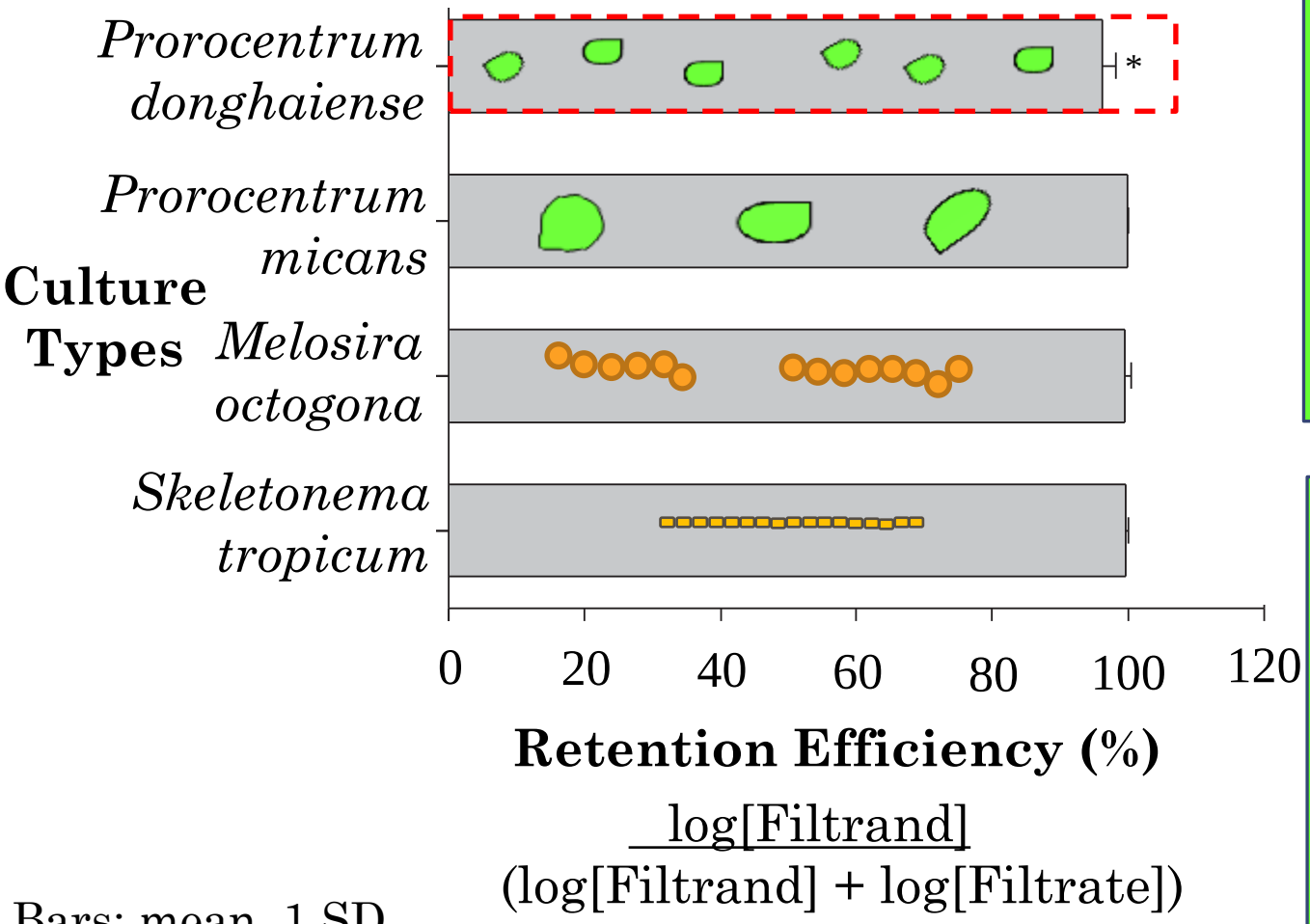
$$\frac{\log[\text{Filtrand}]}{(\log[\text{Filtrand}] + \log[\text{Filtrate}])}$$

***5-µm Nylon** mesh **sig. lower** than all other mesh types ($p<0.001$)	§**5-µm metal** mesh **sig. lower** than the 7-µm Nylon and dual mesh ($p<0.001$) and double mesh ($p = 0.024$)	**No sig. diff. between single, double and dual-stage** filtering using 7-µm or 35-µm Nylon mesh types

Results: Cultured Microalgae RE

Calculated as: **Filtrand divided by filtrand + filtrate**

Note: Single-stage, 7-µm Nylon mesh

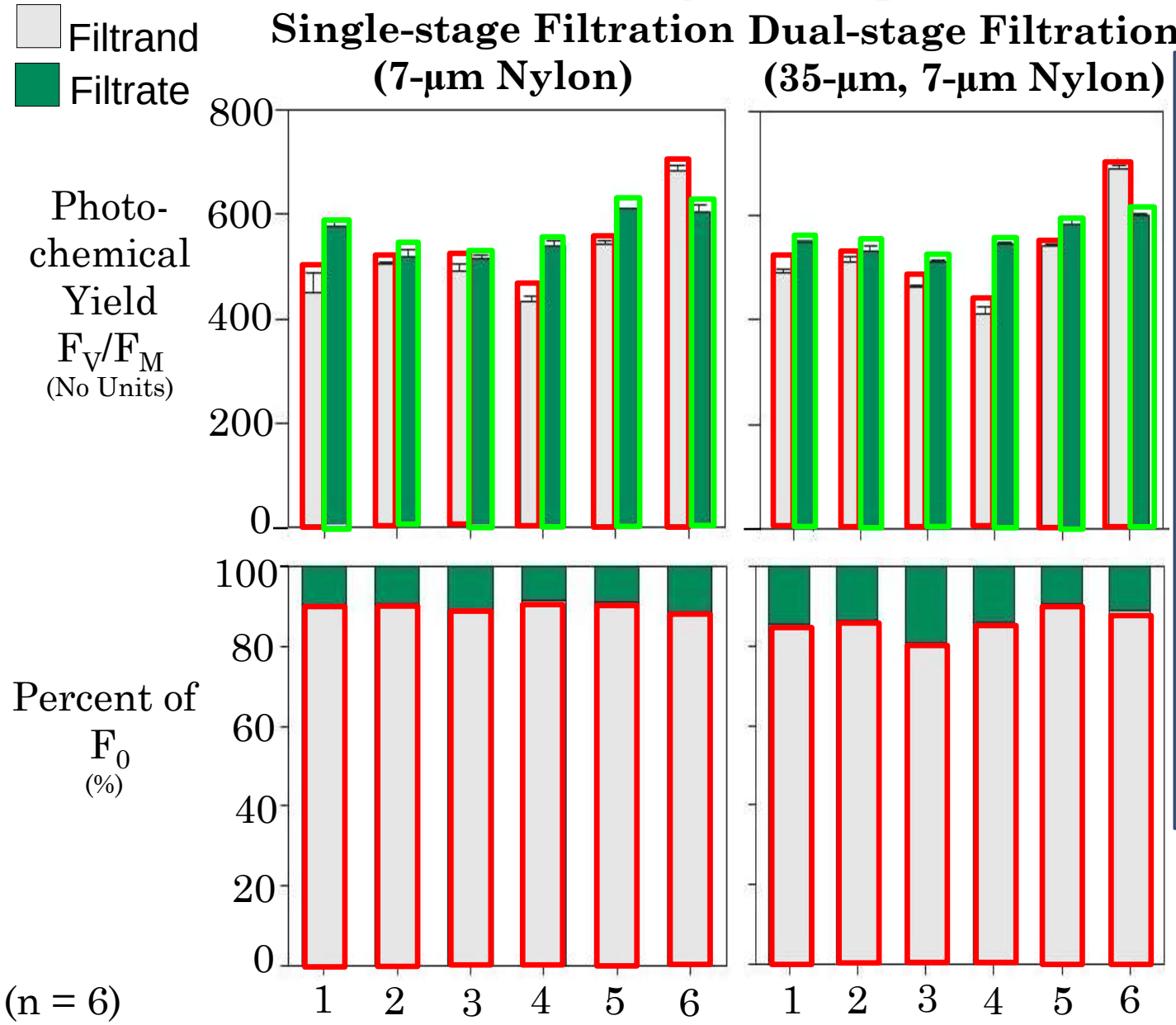


Bars: mean, 1 SD
(n = 6)

- Retention efficiency (RE) **96-100%** using Filtrand + Filtrate calculation

P. donghaiense* **sig. lower than all other microalgae cultures ($p<0.001$)

Results: Ambient Physiological Changes



(n = 6)

Bars: mean, 1 SD

No sig. diff. in the mean F_V/F_M between orgs. in:

- Filtrand and filtrate per trial
- Filtrands of single-stage and dual filtration samples
- Filtrates of single-stage and dual filtration samples

- 7-µm filtrand 80-90% of total initial F_0 signal

Conclusions: Living, Ambient Organism RE

- **Ambient Organisms:**
  - **7-µm Nylon** mesh = **Highest observed RE** (95%)
  - **No significant difference in RE** for all methods **using 7-µm Nylon mesh** (i.e., single mesh, a double-stacked mesh, or filtered through a dual-stage, 35-µm mesh)
  - **5-µm Nylon** mesh = **significantly lower RE** than all other meshes); Possible causes:
    - Lower % of open area = higher flow pressures = "squeezing" animals through holes
    - Increased "embedding" of organisms in 3D mesh structure (not removed via rinsing)

Conclusions: Living, Cultured Microalgae RE

- **Laboratory Cultures:**
  - The **7-µm Nylon mesh** had **RE of >99%** for three out of the four microalgae stocks
    - **Lower RE (96%)** may occur with unicellular organisms **near the 10-µm size threshold** (as seen with *P. donghaiense*)
  - Chain-forming species exhibited relatively **strong chain-retention** (cell recovery in the filtrand [>99%])

Conclusions: Ambient Physiological Changes

- **Ambient Organisms:**
  - **No significant physiological changes** (i.e., changes in fluorescence) were recorded when **comparing photochemical yield** (F_V/F_M) among all filtration configurations

  - For F_0 calculations combining filtrand and filtrate signals:
    - 7-µm filtrand represented 80%-90% of the total signal (ratio comparable to organism [≥10 and <50 µm size] retention in filtrand as measured by manual microscopy)

Final Conclusions

- Based on the findings,
  - Four filter configurations comparable to use in concentrating ≥10 and <50 µm organisms for live counts and variable fluorescence measurements
    1. double-stacked 7-µm Nylon
    2. 35-µm dual-stage Nylon filtered
    3. single 7-µm Nylon
    4. 5-µm stainless steel with the exclusion of 5-µm Nylon
- Single-stage, 7-µm Nylon specifically recommended based on ease of use

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Supplemental Slides

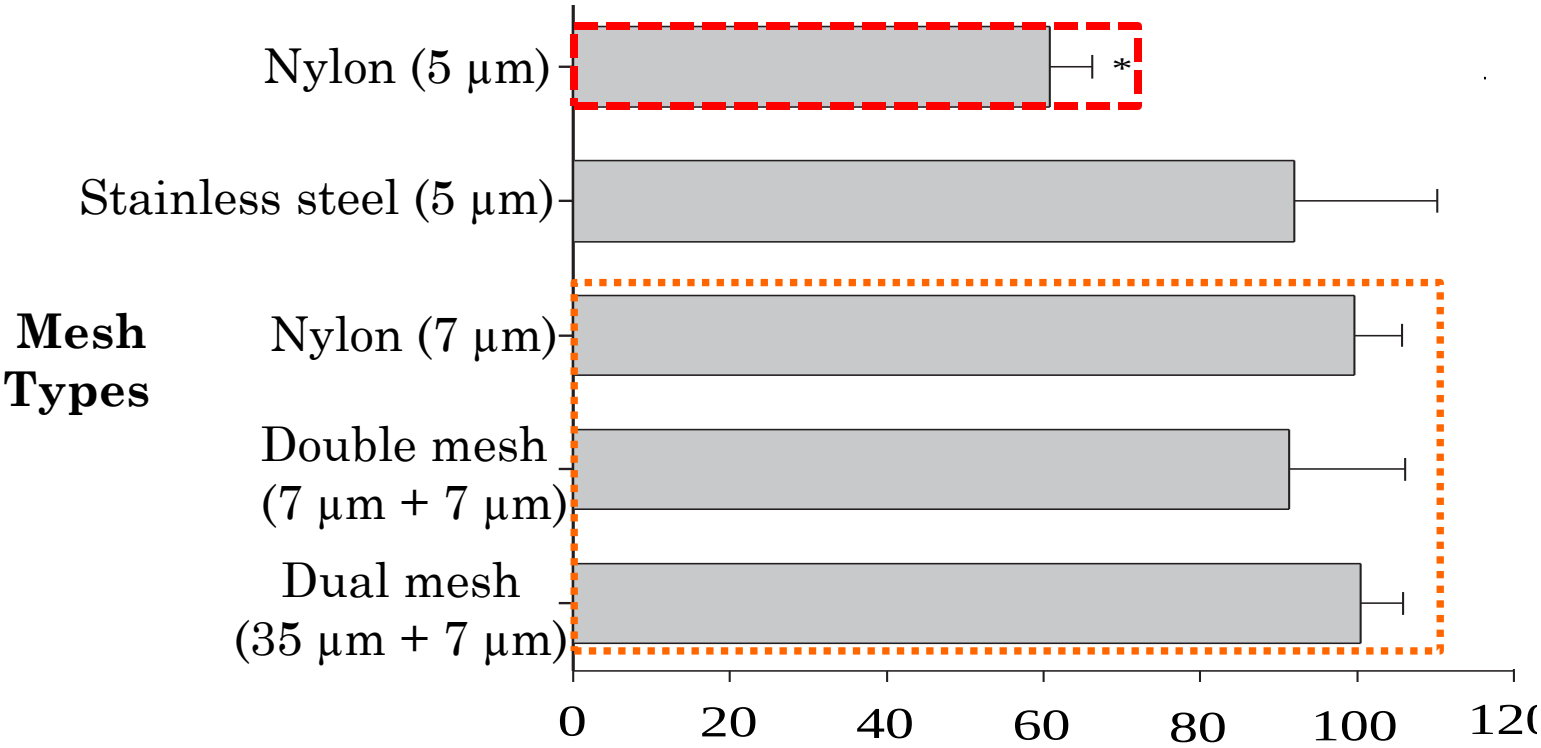
Laboratory Cultured Microalgae Stock

Cell Dimensions
(min. to max. range)

Name	Length (µm)	Width (µm)	Chain-former	Image
Prorocentrum donghaiense	12-16	10-14	No	
Prorocentrum micans	28-48	14-30	No	
Skeletonema tropicum	5-10	8-10	Yes	
Melosira octogona	16-24	14-26	Yes	

Results: Living, Ambient Organism RE

Calculated as: **Filtrand divided by whole water**



Bars = mean, 1 SD
n = 3 to 6

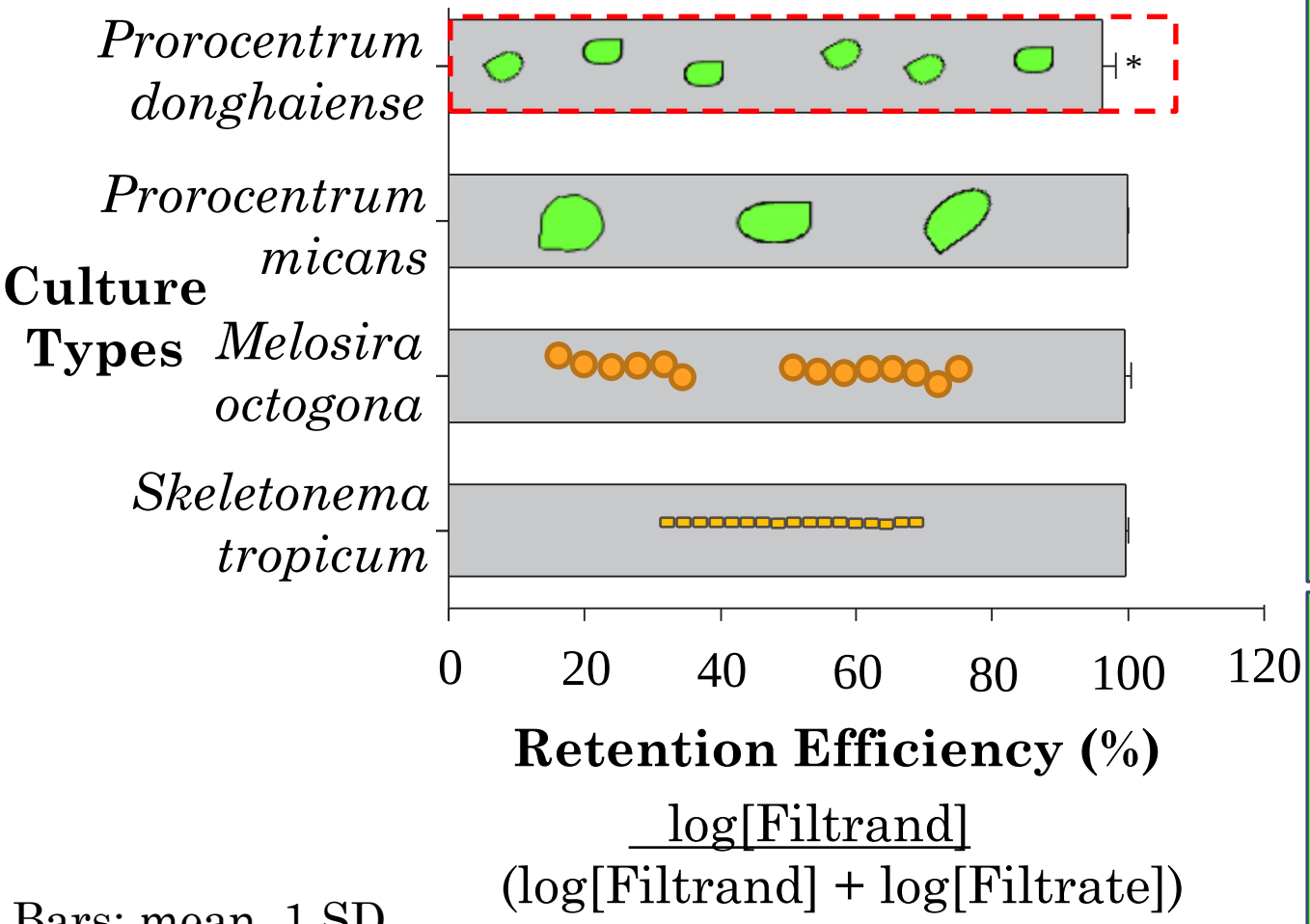
***5-µm Nylon mesh** retention efficiency (RE) **sig. lower** than other mesh types ($p<0.001$)

No sig. diff. in RE between single, double and dual-stage filtering when using 7-µm or 35-µm Nylon mesh

Results: Cultured Microalgae RE

Calculated as: **Filtrand divided by filtrand + filtrate**

Note: Single-stage, 7-µm Nylon mesh



Bars: mean, 1 SD
(n = 6)

- Retention efficiency (RE) **96-100%** using Filtrand + Filtrate calculation
- RE ranged from **93-102%** using whole water calculation (data not shown)

P. donghaiense* **sig. lower than all other microalgae cultures ($p<0.001$) (not the case for whole water calculations)

Next Steps

- Filtration trials on additional mesh types
  - Etched metal mesh
  - Chemically-etched membrane filter (advantage: lacks 3D structure)
  - Smaller pore sized nylon mesh (e.g., 3-µm nylon)

- Examination of organisms remaining on filtration meshes
  - DNA extraction and identification