

UNIFORM RESPONSE OF ORGANISMS IN DIFFERENT PHYLOGENETIC GROUPS AND SIZE CLASSES TO BALLAST WATER TREATMENTS

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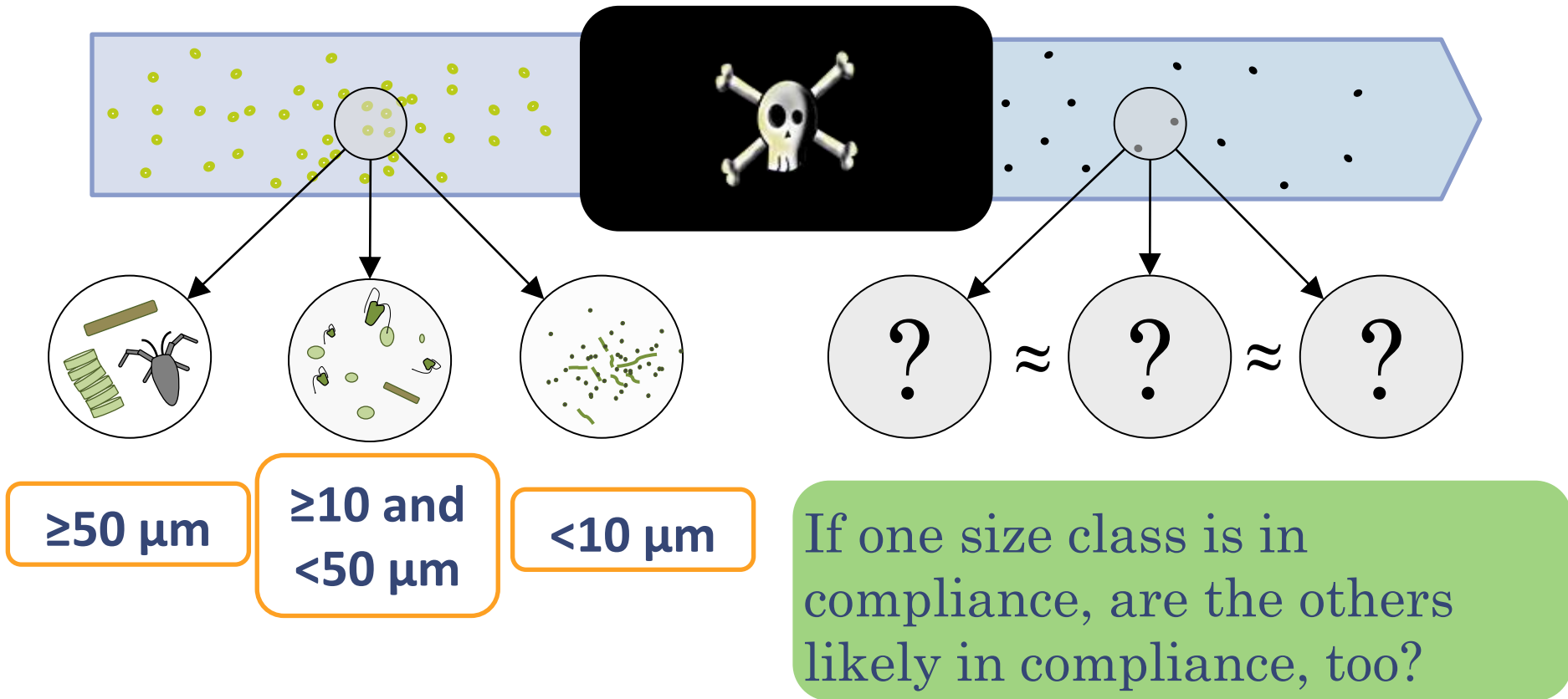
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Background: Treatment and Size Classes



Goal: Rapid compliance testing; examine one size class to predict the response of others

Research Goals

- Examine the response of organisms in the three size classes to treatments
- Mimic treatment used in ballast water management systems (chlorination, ultraviolet [UV] radiation, and deoxygenation)
- Examine the responses of organisms across a range of doses

Treatment Types

○ Chlorination

Hypochlorite produced via electrolysis of seawater

○ UV radiation

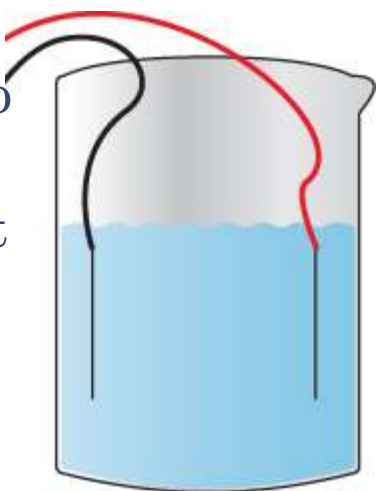
UV radiation delivered via a collimated beam apparatus

○ Deoxygenation

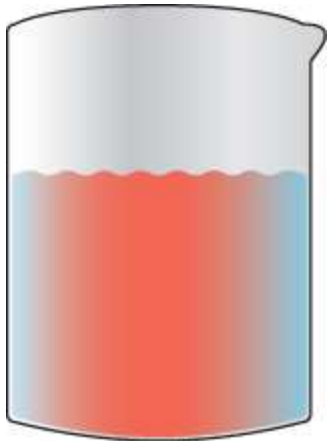
Oxygen (O_2) displacement by bubbling nitrogen (N_2) gas

Treatment: Chlorination

Electrodes connected to a potentiostat



Electrochlorination



Hypochlorite Stock solution

Hypochlorite was added to seawater with organisms



Concentrations of free chlorine measured with a colorimetric approach



Hach, Inc; Loveland, CO

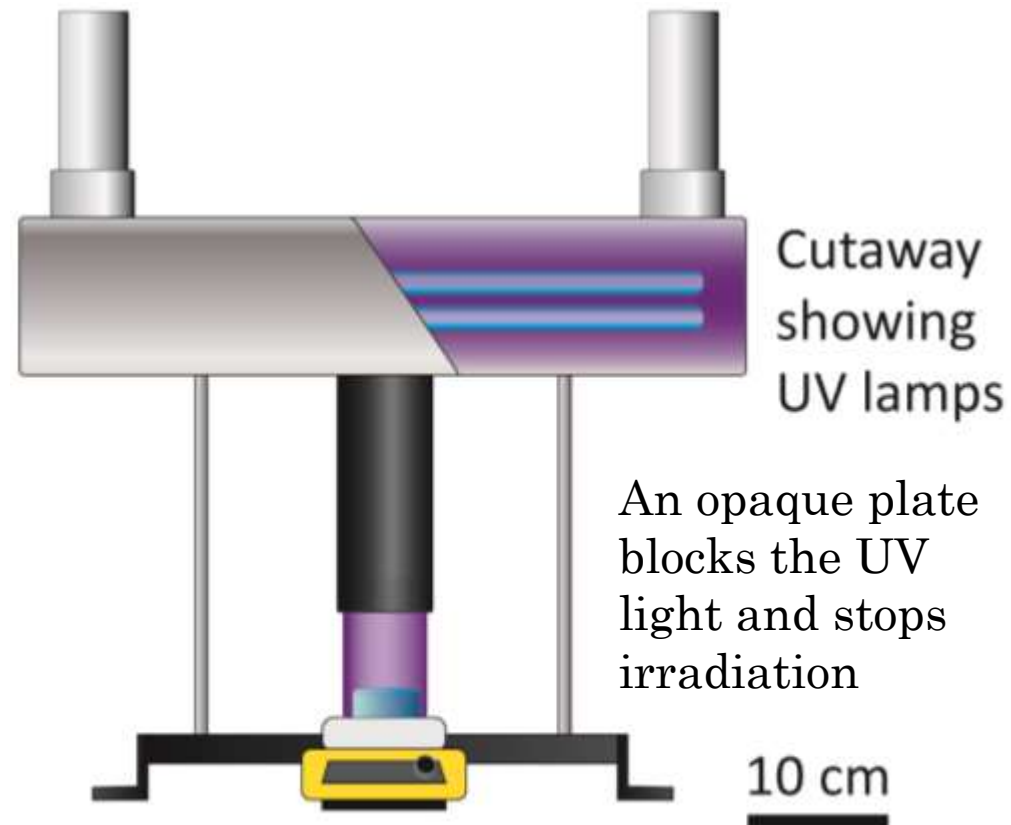
Seawater Characteristics:
Temp.: 20 – 24°C
Salinity: 36 – 37 psu
pH: 8.4

Target concentrations were 7.28 (low) and 12.28 (high) mg L⁻¹

Treatment: UV Radiation

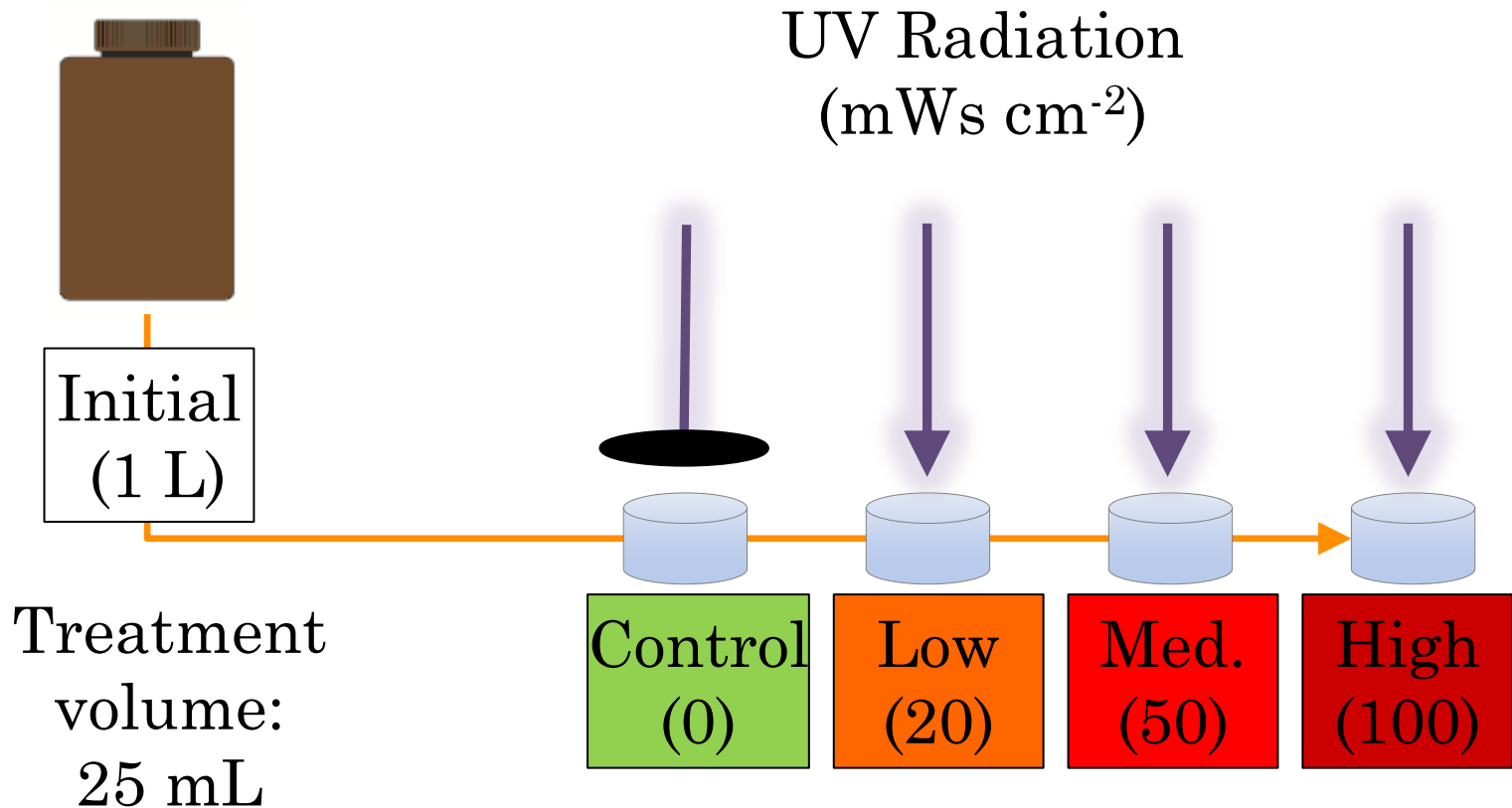
- UV Collimated Beam
- Delivers an exact dose of UV radiation
- Sample (25 mL) stirred continuously during exposure

Dose (mWs cm ⁻²)	Exposure time (min:sec)
0	0
20	2:18
50	5:45
100	11:30



- Irradiation (i.e., fluence) is constant
- Thus, dose is controlled by exposure time
Dose = fluence x time

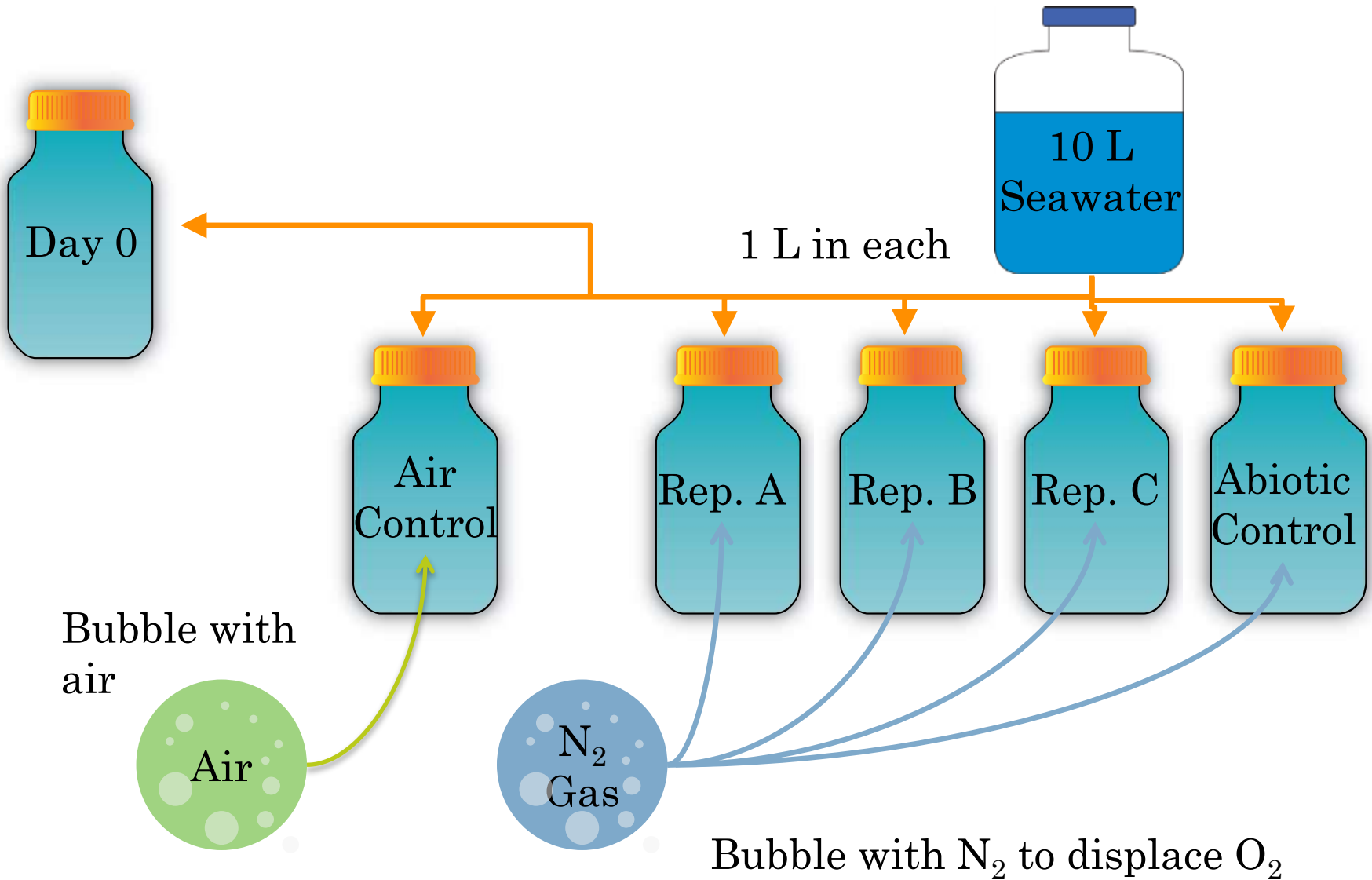
Treatment: UV Radiation



Analysis at three time points for each treatment:

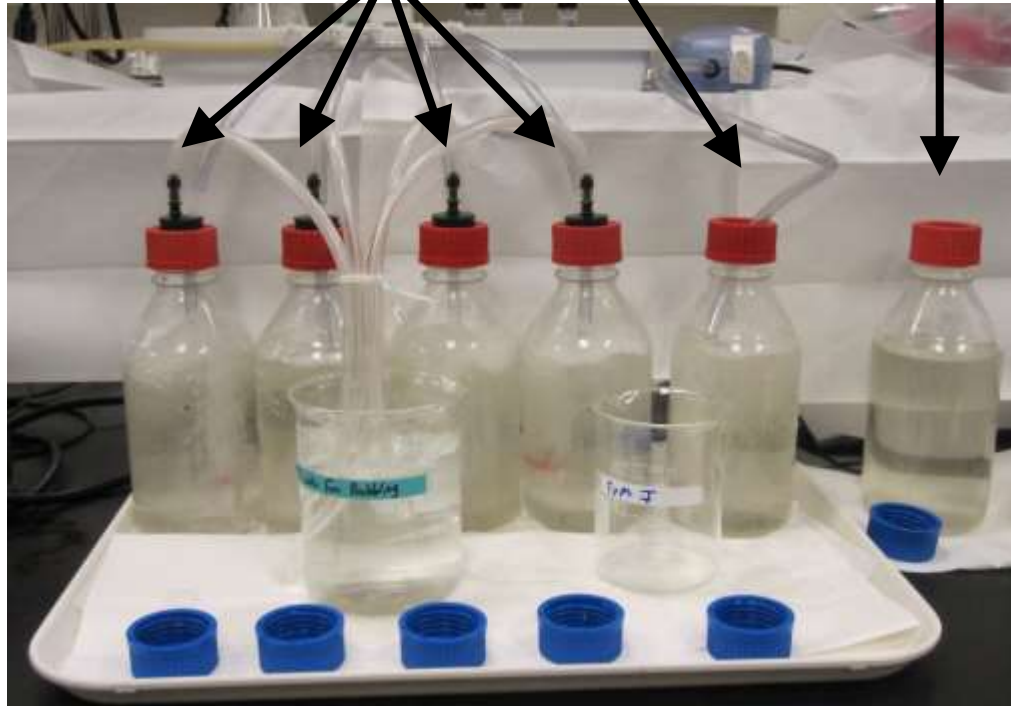
- Day 0 (Initial Analysis)
- Day 1
- Day 5

Treatment: Deoxygenation



Treatment: Deoxygenation

N₂ gas Air Initial analysis O₂ Analysis



Optical O₂
Probe



O₂ Titration
Kit

A concentrated suspension of ambient organisms was added to each bottle following treatment

Deoxygenation: O₂ Concentrations

Treatment	Oxygen Concentration (mg L ⁻¹)
Initial	8.1 ± 0.2
Air Control	5.0 ± 1.9
Abiotic Control	1.6 ± 2.8
Rep. A	0.18 ± 0.03
Rep. B	0.17 ± 0.03
Rep. C	0.13 ± 0.05

Values: mean ± 1 SD (n = 5)

Experimental Design: Test Organisms

Goal: Prepare a sample to meet Environmental Technology Verification (ETV) challenge water criteria

$\geq 50 \mu\text{m}$ Organisms

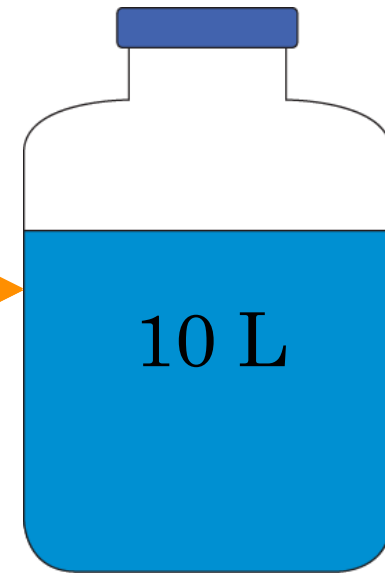
100 L^{-1} ($100,000 \text{ m}^{-3}$) of concentrated ambient organisms

≥ 10 and $< 50 \mu\text{m}$ Organisms

$1,000 \text{ mL}^{-1}$ of concentrated ambient organisms amended with cultured microalgae

ETV Additives

Challenge water concentrations of dissolved and particulate matter



Initial counts of organisms performed to verify concentrations

Note:

Ambient concentrations of $< 10 \mu\text{m}$ org.

Sample Analysis

Size Class	Approach	Description
$\geq 50 \mu\text{m}$	Manual counting; brightfield microscopy	5-mL Bogorov chamber
≥ 10 and $< 50 \mu\text{m}$	Manual counting; vital staining and epifluorescence microscopy	1-mL Sedgewick-Rafter chamber
≥ 10 and $< 50 \mu\text{m}$	Variable fluorescence	Pulse Amplitude Modulated (PAM) fluorometry
$< 10 \mu\text{m}$	Heterotrophic plate counts; culturing aerobic bacteria on nutrient agar	Colony forming units (CFU) observed after a 1 and 5-d incubation

Chlorination Results

At 12 mg L⁻¹, concentrations of >10 and <50 μm

Repopulation of heterotrophic bacteria at day 5

Concentrations of Living Organisms

..... ETV Challenge Water
 Discharge Std.

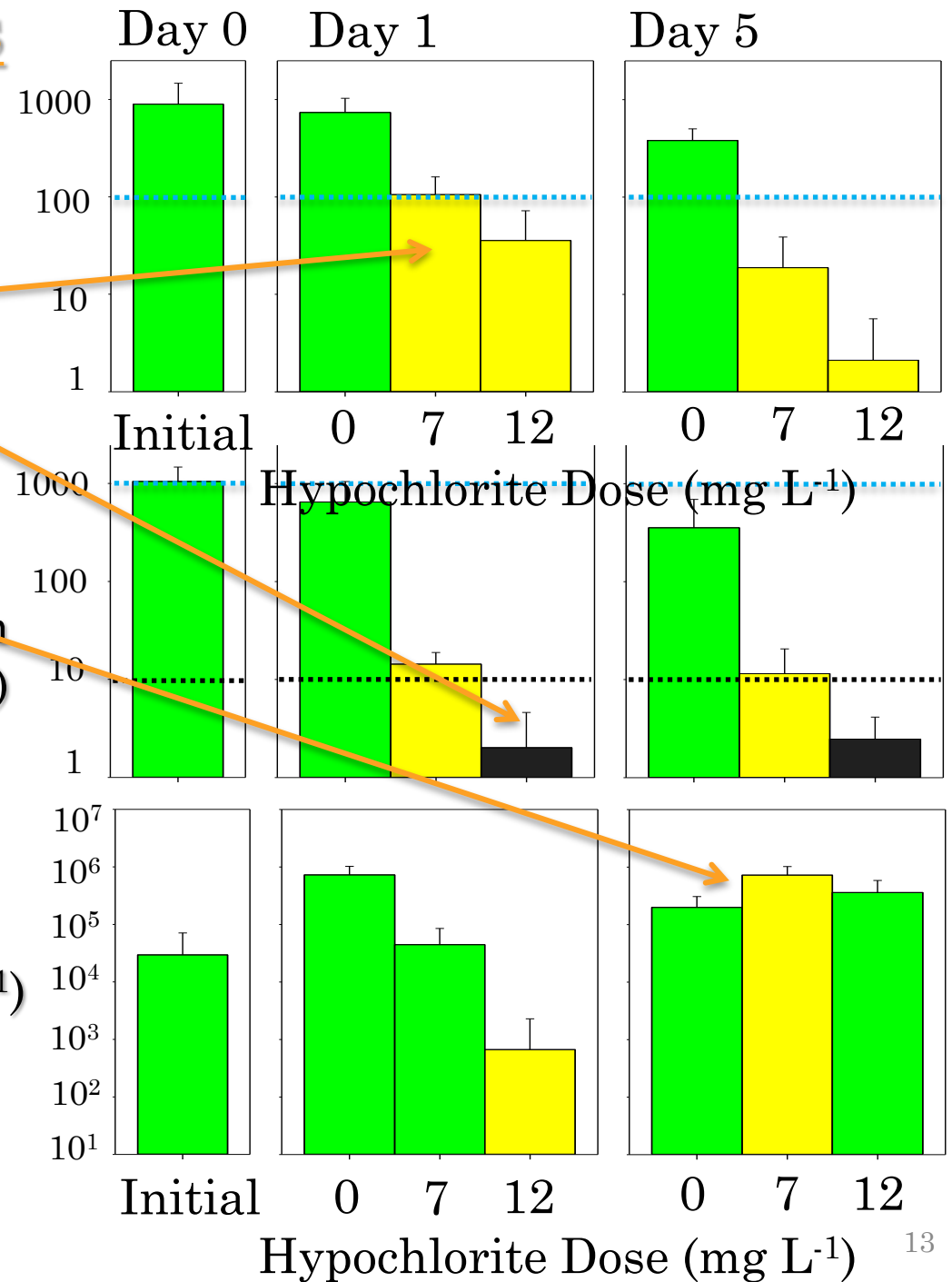
■ Not Sig. Diff. from Control
 ■ Sig. Diff. from Control
 ■ Sig. Less than Discharge Std.

Bars: mean ± 1 SD

≥50 μm (L⁻¹)

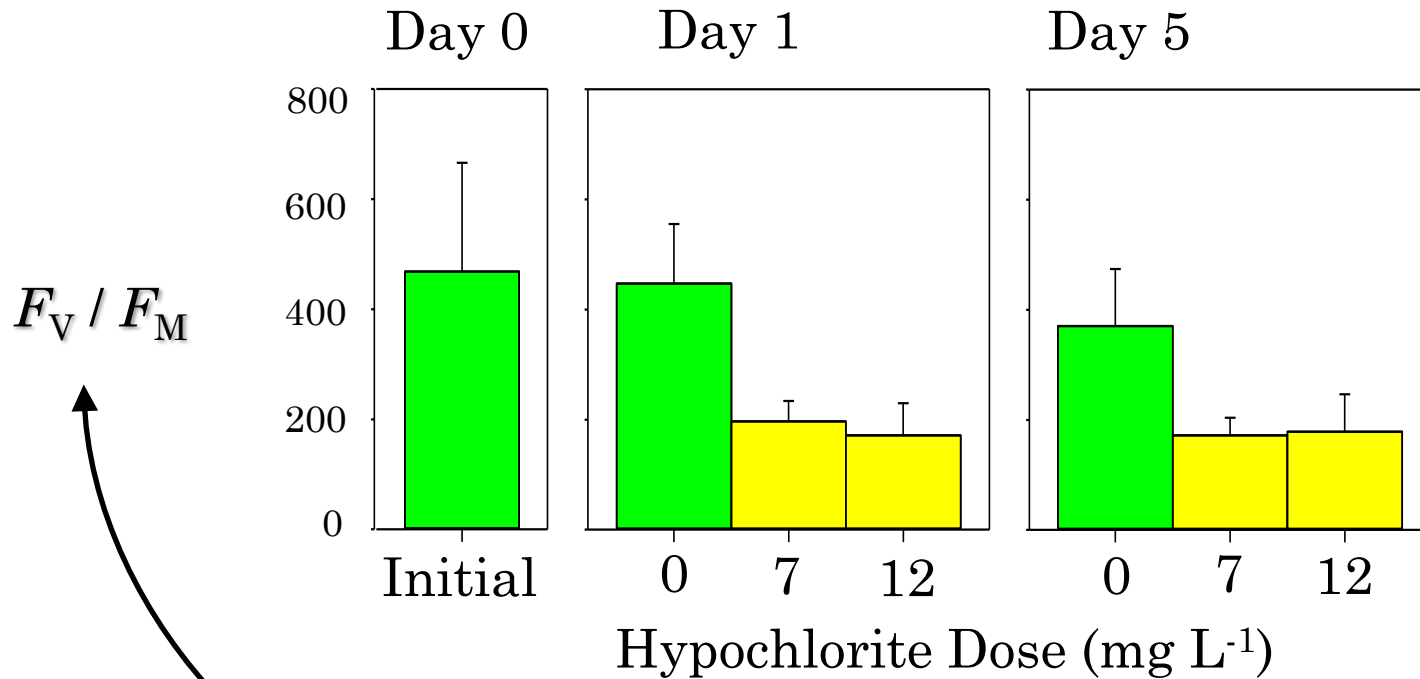
≥10 and <50 μm (mL⁻¹)

CFU (log mL⁻¹)



Chlorination Results: Variable Fluorescence

F_V / F_M in 7 and 12 mg L⁻¹ treatments were **significantly different** from control both at day 1 and day 5



- Not Sig. Diff. from Control
- Sig. Diff. from Control

- An indicator of physiological status of algae
- No units; potential range: 0 – 1,000
- Measurements of living communities typically >400

UV Radiation Results

Day 0

Day 1

Day 5

Significant decrease in bacteria at 50 and 100 mWs cm⁻², but only immediately after treatment

Concentrations of Living Organisms

$\geq 50 \mu\text{m}$
(L⁻¹)

≥ 10 and $< 50 \mu\text{m}$
(mL⁻¹)

..... ETV Challenge Water

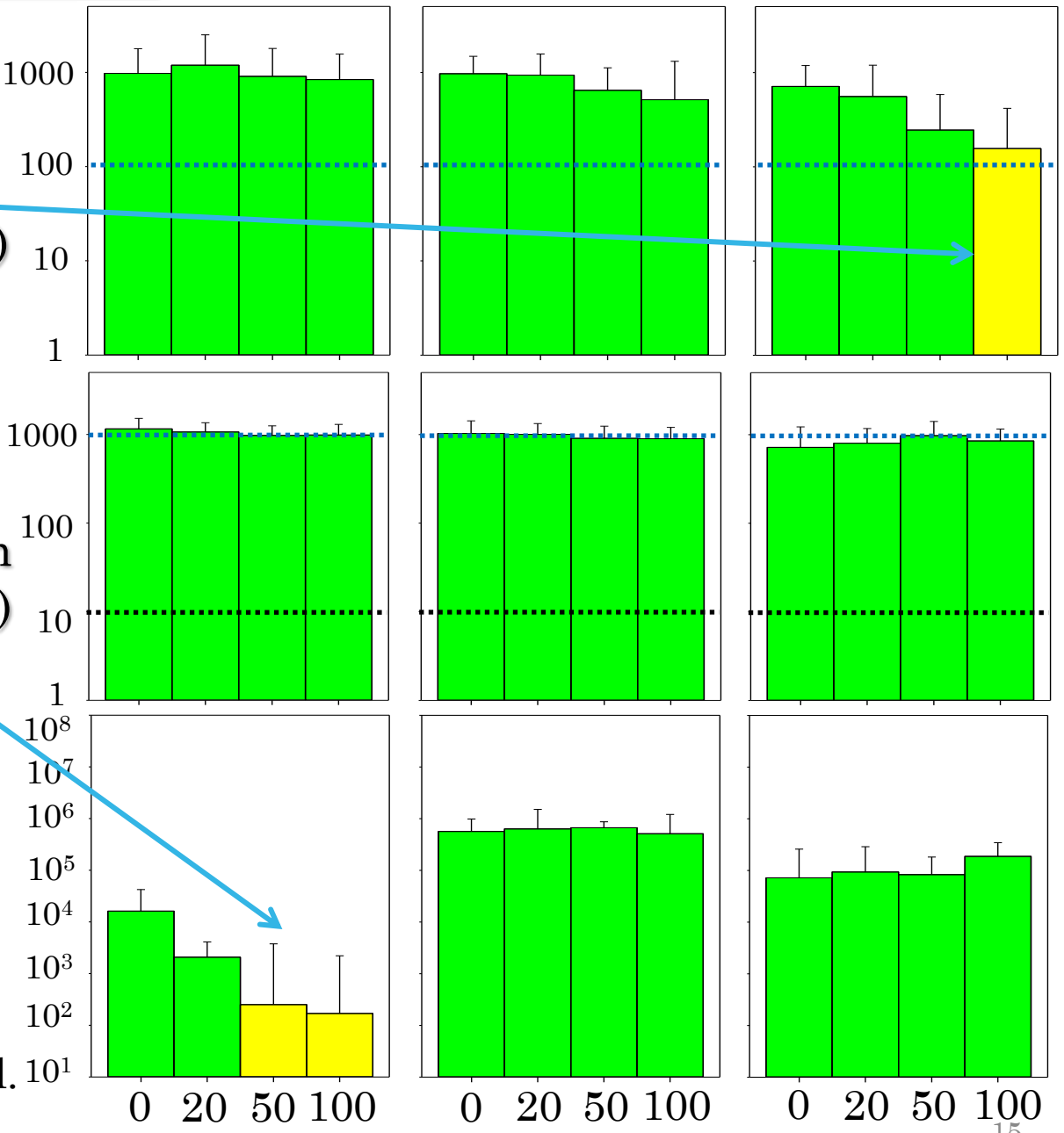
..... Discharge Std. CFU
(log mL⁻¹)

■ Not Sig. Diff. from Control

■ Sig. Diff. from Control

■ Sig. Less than Discharge Std.

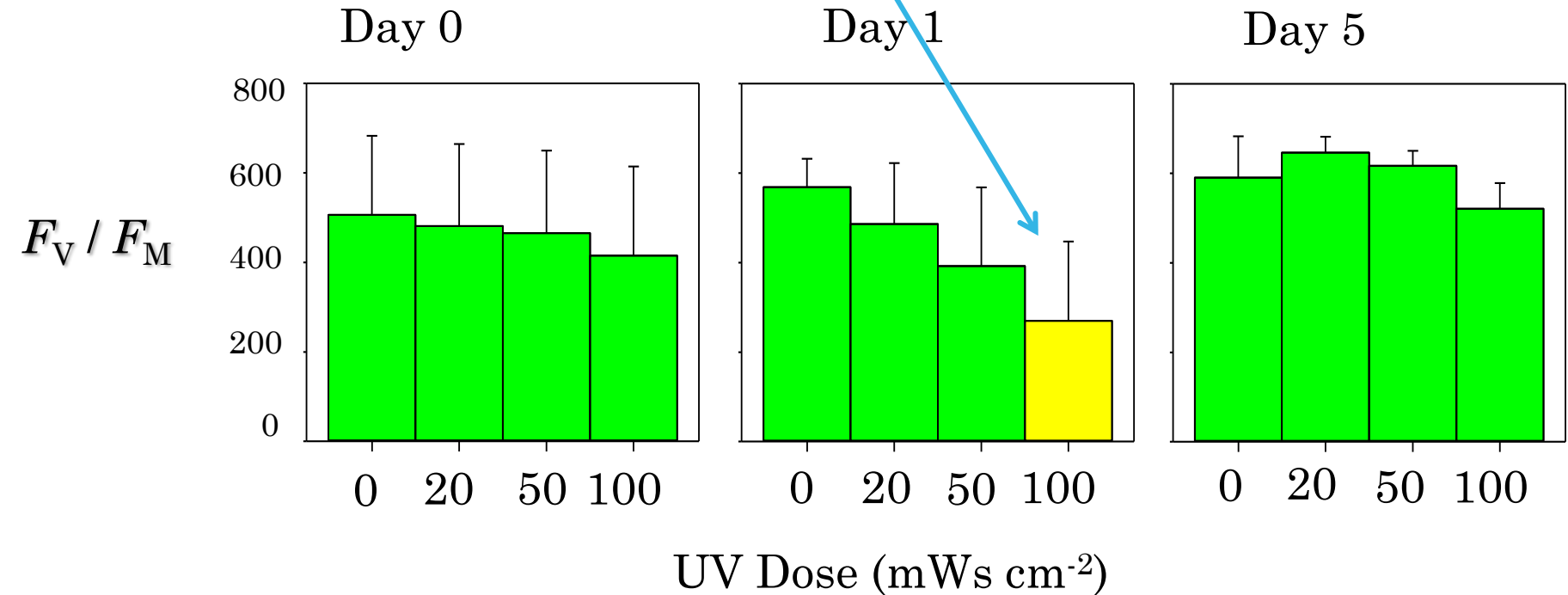
Bars: mean \pm 1 SD



UV Dose (mWs cm⁻²)

UV Radiation Results: Variable Fluorescence

- High variation in F_V/F_M measurements
- Only at **100 mWs cm⁻²** were readings significantly different from other treatments and only at day 1



- Not Sig. Diff. from Control
- Sig. Diff. from Control

Bars: mean \pm 1 SD

Deoxygenation Results

Heterotrophic bacteria: $\geq 50 \mu\text{m} \text{ (L}^{-1}\text{)}$
No significant difference in concentrations in controls or treatment subsamples

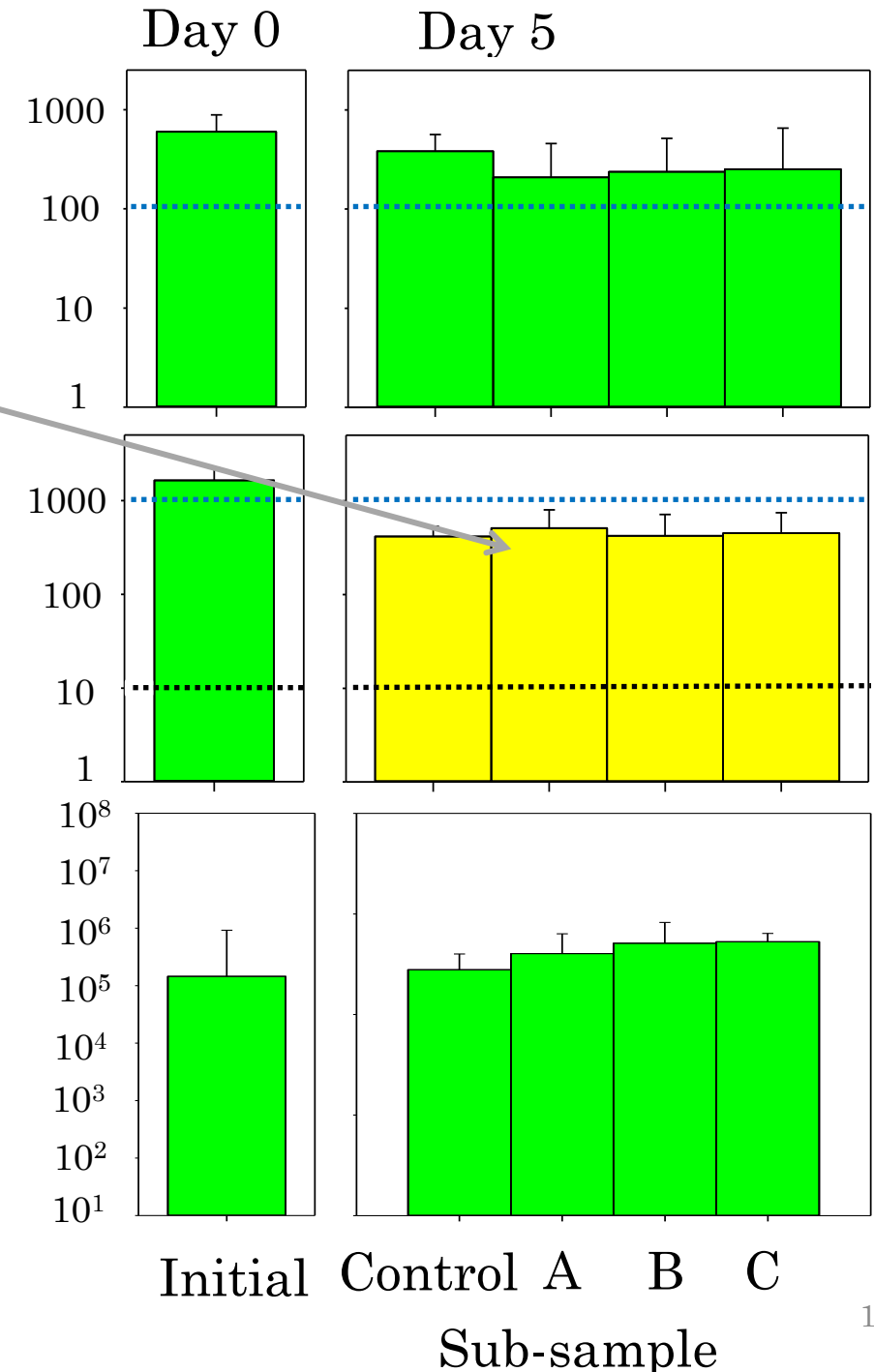
Concentrations of Living Organisms ≥ 10 and $< 50 \mu\text{m} \text{ (mL}^{-1}\text{)}$

..... ETV Challenge Water
 Discharge Std.

CFU $\text{(log mL}^{-1}\text{)}$

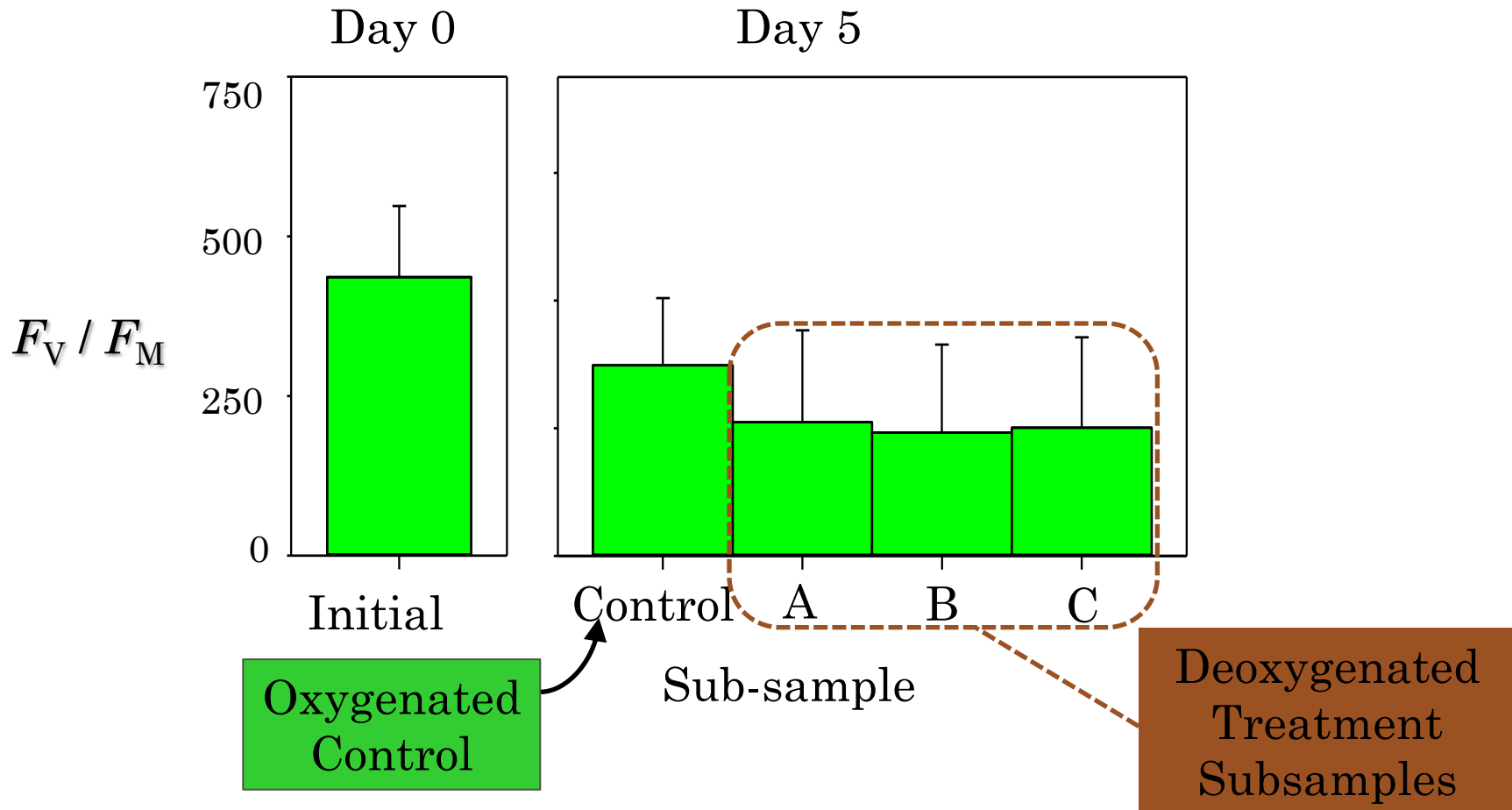
■ Not Sig. Diff. from Control
 ■ Sig. Diff. from Control
 ■ Sig. Less than Discharge Std.

Bars: mean \pm 1 SD



Deoxygenation Results: Variable Fluorescence

F_V / F_M measurements **not significantly different** from control at day 5 in any of the three subsample treatments



- Not Sig. Diff. from Control
- Sig. Diff. from Control

Bars: mean \pm 1 SD

Conclusions

- **Chlorination:** Significant reductions in organism concentrations relative to the control for all size classes at day 1 for both 7 and 12 mg L⁻¹ treatments
- **UV Radiation:** Significant reductions in concentrations of heterotrophic bacteria (relative to control) immediately after irradiation (50 and 100 mWs cm⁻²)
- **Deoxygenation:** Significant differences in live organism concentrations were not observed in any size class (relative to control)
- Generally, organisms $\geq 50 \mu\text{m}$ and ≥ 10 and $< 50 \mu\text{m}$ showed similar responses to treatment, although the magnitude of their responses varied
- Because most BWMS employ a filtration step to remove organisms $\geq 50 \mu\text{m}$, if the organisms in the ≥ 10 and $< 50 \mu\text{m}$ size class are assessed to provide an indication of a vessel's compliance with the discharge standard, it would be imperative to ensure the filter was functioning properly and removed organisms as designed

Acknowledgements

This work was funded by the US Coast Guard (USCG) Environmental Standards Division (CG-OES-3)
(MIPR HSCG23-13-X-MMS106)

We thank **Richard Everett** and **Regina Bergner** for their advice and programmatic support

This work does not represent the official position of the USCG

This work was supported by
Diane Lysogorski and **Elizabeth Hogan**,
Former and Acting Section Heads, respectively, of
NRL Code 6136 and Directors of the
Center for Corrosion Science and Engineering –
Key West, Florida

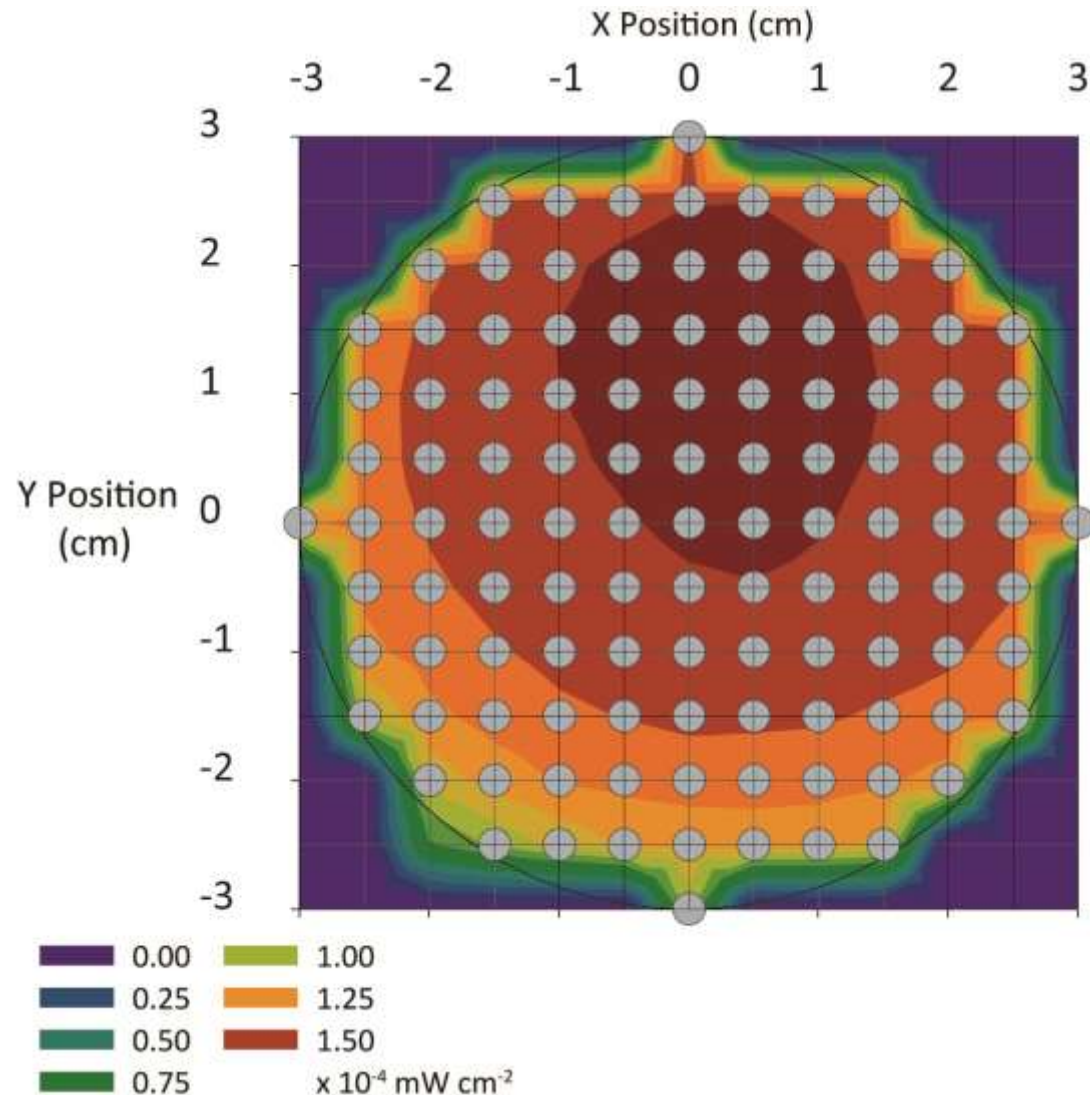
SUPPLEMENTAL SLIDES

Chemistry Division, Naval Research Laboratory, Washington, DC 23075

Treatment: UV Radiation

Incident irradiation is a function of:

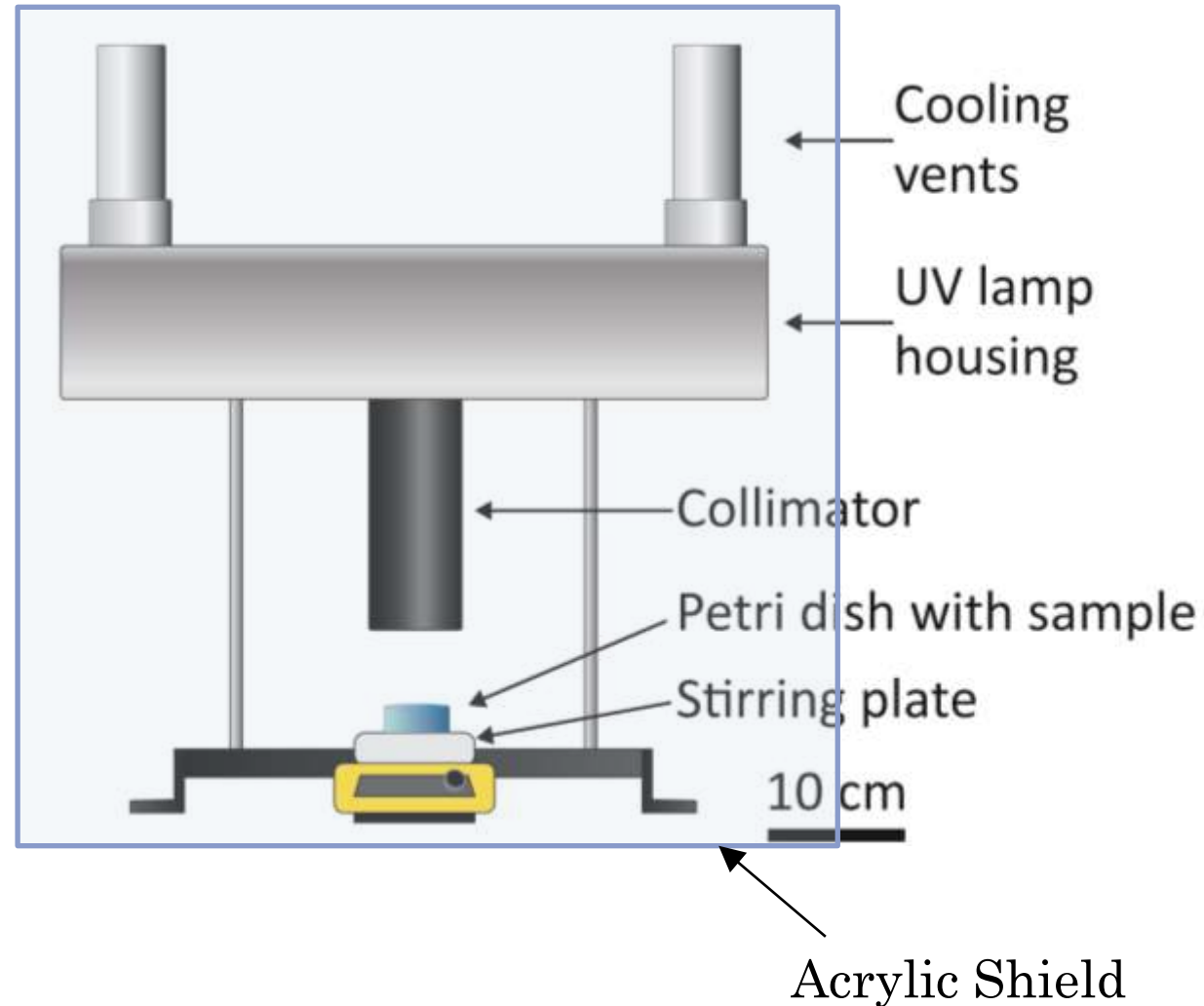
1. Surface reflectance
2. Water depth
3. Absorption
4. Length of light path
5. Petri factor
(A map of fluence values across the area of a Petri dish)



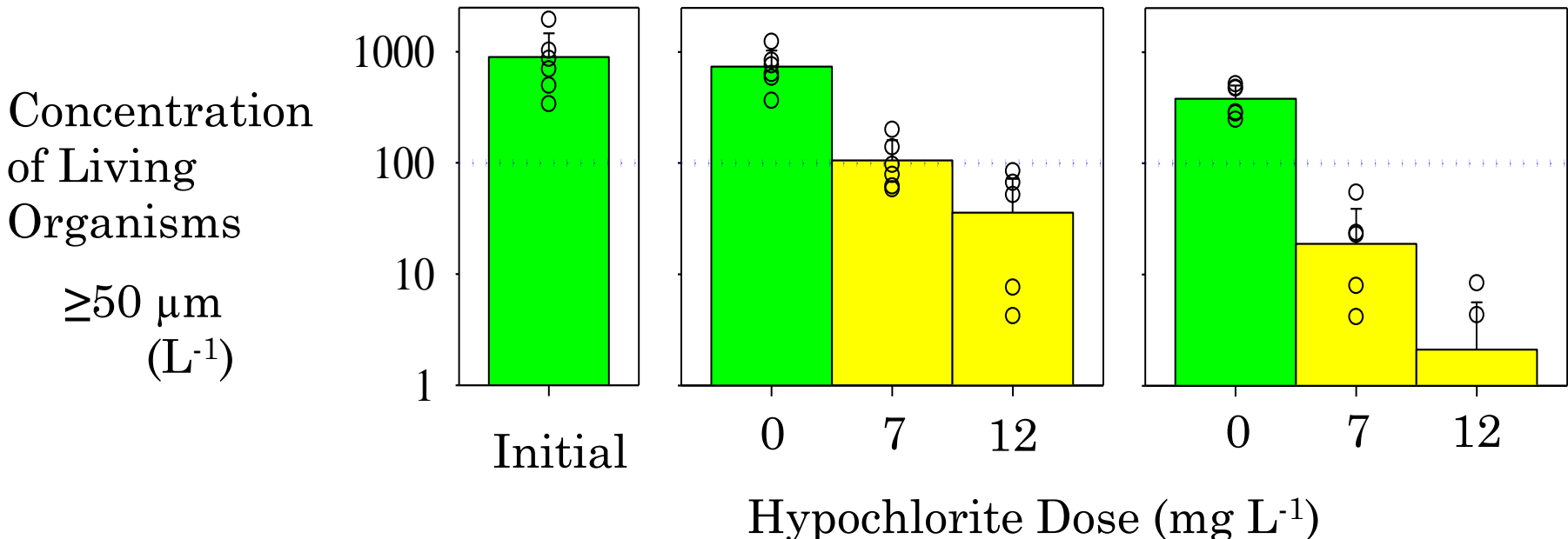
Calculated fluence: 15.4 mW cm^{-2}

Treatment: UV Radiation

- UV Collimated Beam
- Delivers an exact dose of UV radiation
- Sample (25 mL) stirred continuously during exposure



Chlorination Results: Organisms $\geq 50 \mu\text{m}$



Concentrations are significantly reduced at both 7 and 12 mg L^{-1} after day 1

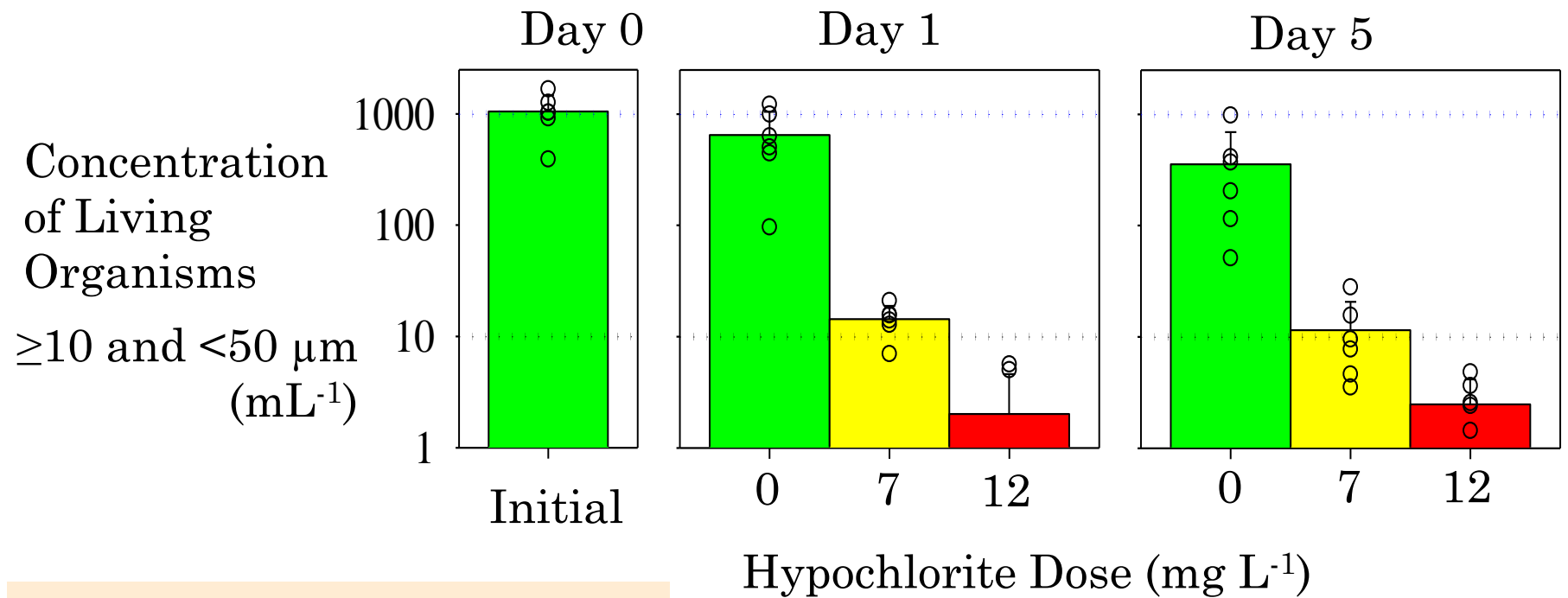
■ Control
■ Sig. Diff. from Control
■ Sig. Less than Discharge Std.

⋯⋯⋯ ETV Challenge Water - - - - - Discharge Std.

Bars: mean \pm 1 SD

(n = 6; shown as symbols on the bars)

Chlorination Results: Organisms ≥ 10 and $< 50 \mu\text{m}$



Analysis:
 Epifluorescence microscope counts of organisms labeled with vital stains (Steinberg et al., 2011)

In 12 mg L^{-1} , concentrations of ≥ 10 and $< 50 \mu\text{m}$ sig. less than the discharge std. (t-test, $p > 0.05$)

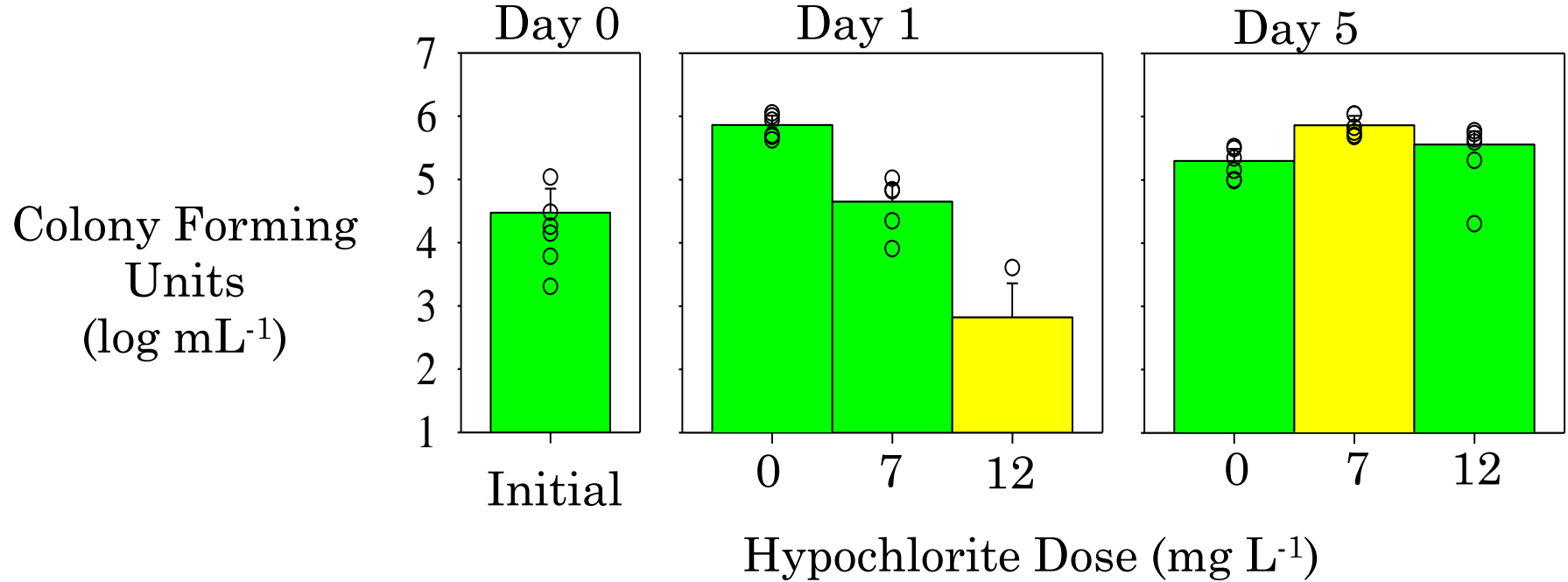
..... ETV Challenge Water Discharge Std.

■ Control ■ Sig. Diff. from Control ■ Sig. Less than Discharge Std.

Bars: mean \pm 1 SD

(n = 6; shown as symbols on the bars)

Chlorination Results: Heterotrophic Bacteria



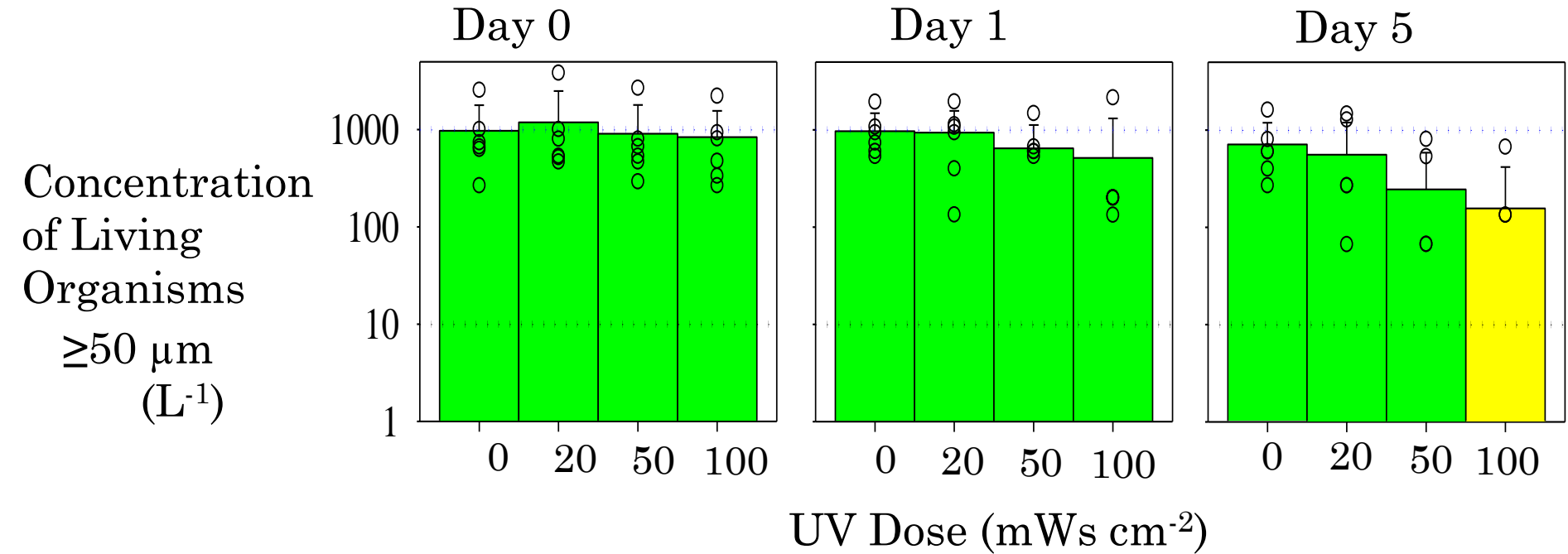
Analysis:
Measurement of
total Heterotrophic
Bacteria

Repopulation of heterotrophic bacteria
after day 5

- Control
- Sig. Diff. from Control
- Sig. Less than Discharge Std.

Bars: mean \pm 1 SD
(n = 6; shown as symbols on the bars)

UV Radiation Results: Organisms $\geq 50 \mu\text{m}$



Only high doses (100 mWs cm^{-2}) significantly reduce concentrations after 5 days (ANOVA, $p < 0.05$)

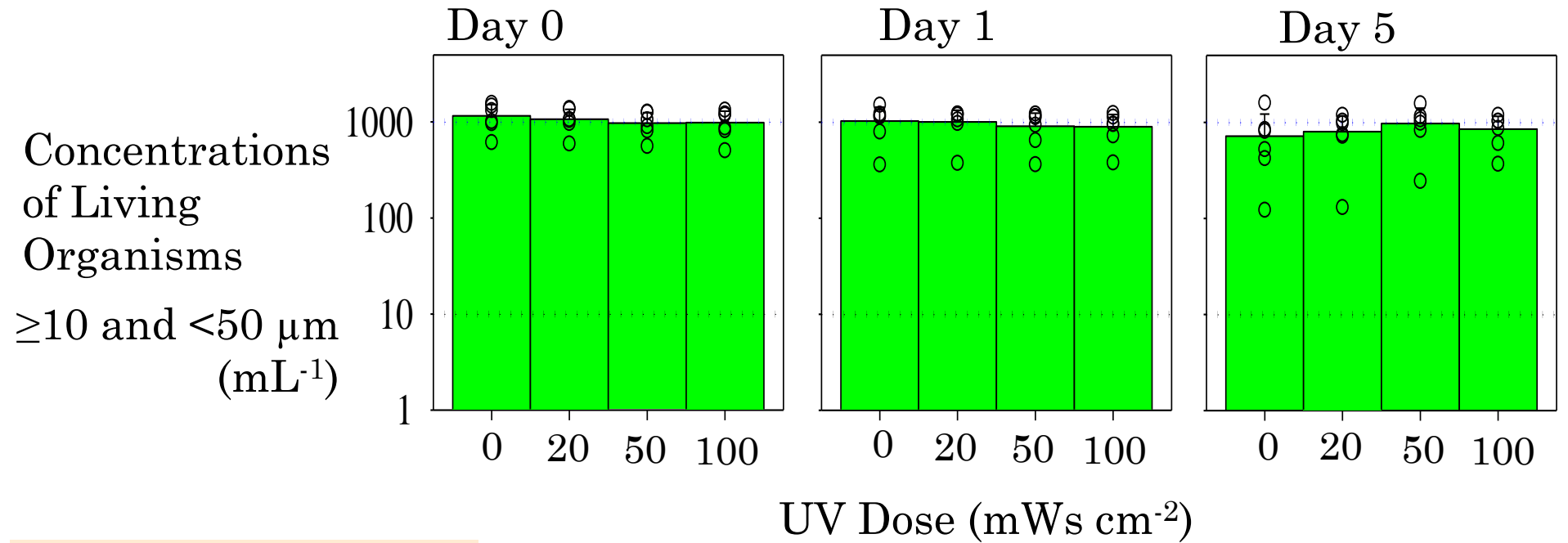
..... ETV Challenge Water Discharge Std.

■ Control ■ Sig. Diff. from Control

■ Sig. Less than Discharge Std.

Bars: mean \pm 1 SD
($n = 6$; shown as symbols on the bars)

UV Radiation Results: Organisms ≥ 10 and $< 50 \mu\text{m}$



Analysis:

Epifluorescence microscope counts of organisms labeled with vital stains

No significant changes in the concentrations of living organisms ≥ 10 and $< 50 \mu\text{m}$ (ANOVA, $p > 0.05$)

..... ETV Challenge Water Discharge Std.

Bars: mean \pm 1 SD

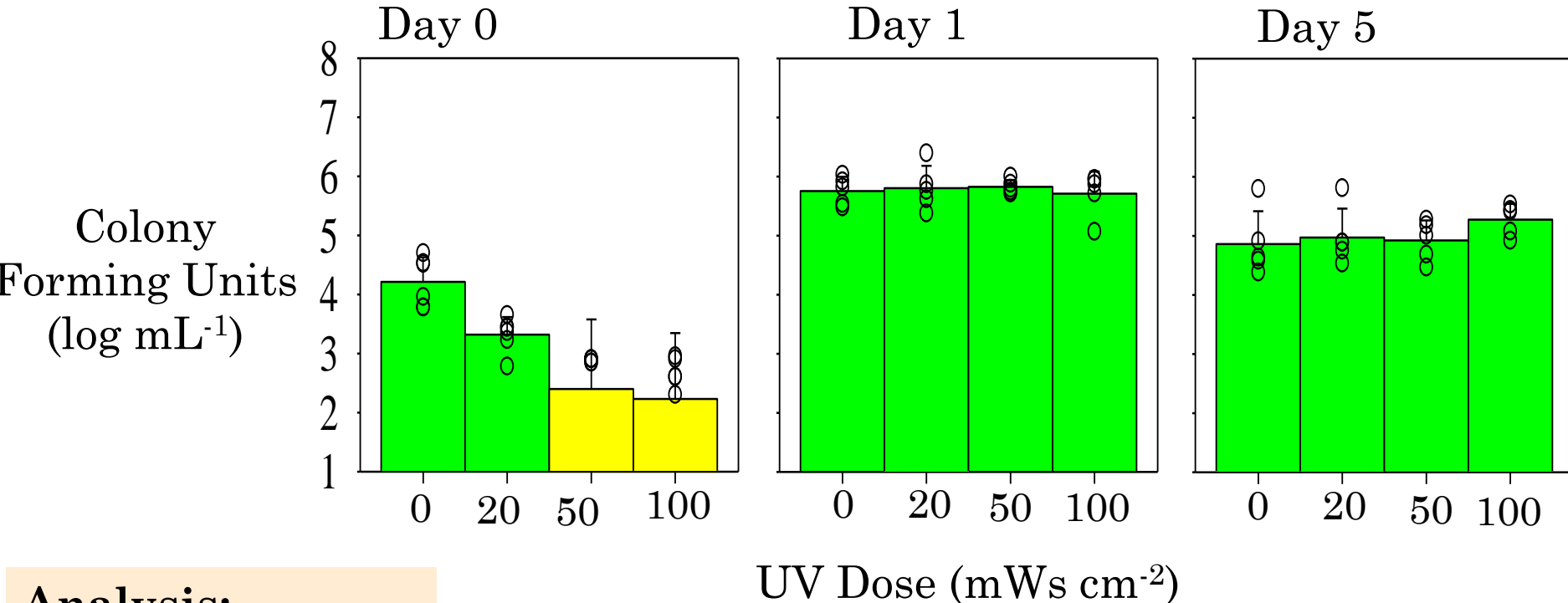
($n = 6$; shown as symbols on the bars)

■ Control

■ Sig. Diff. from Control

■ Sig. Less than Discharge Std.

UV Radiation Results: Heterotrophic Bacteria



Analysis:
 Measurement of total Heterotrophic Bacteria

Significant reduction in bacteria at 50 and 100 mWs cm⁻² (ANOVA, p<0.05), but only immediately after treatment

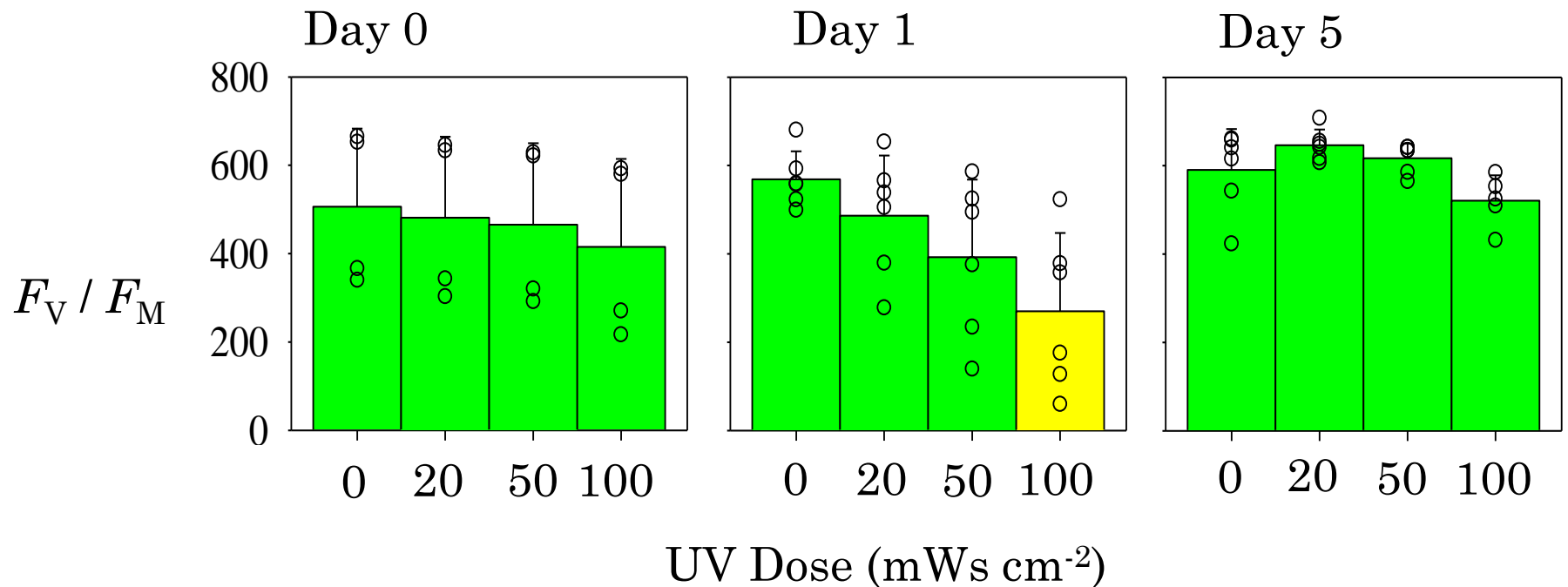
- Control
- Sig. Diff. from Control
- Sig. Less than Discharge Std.

..... ETV Challenge Water Discharge Std.

Bars: mean ± 1 SD
 (n = 6; shown as symbols on the bars)

UV Radiation Results: Variable Fluorescence

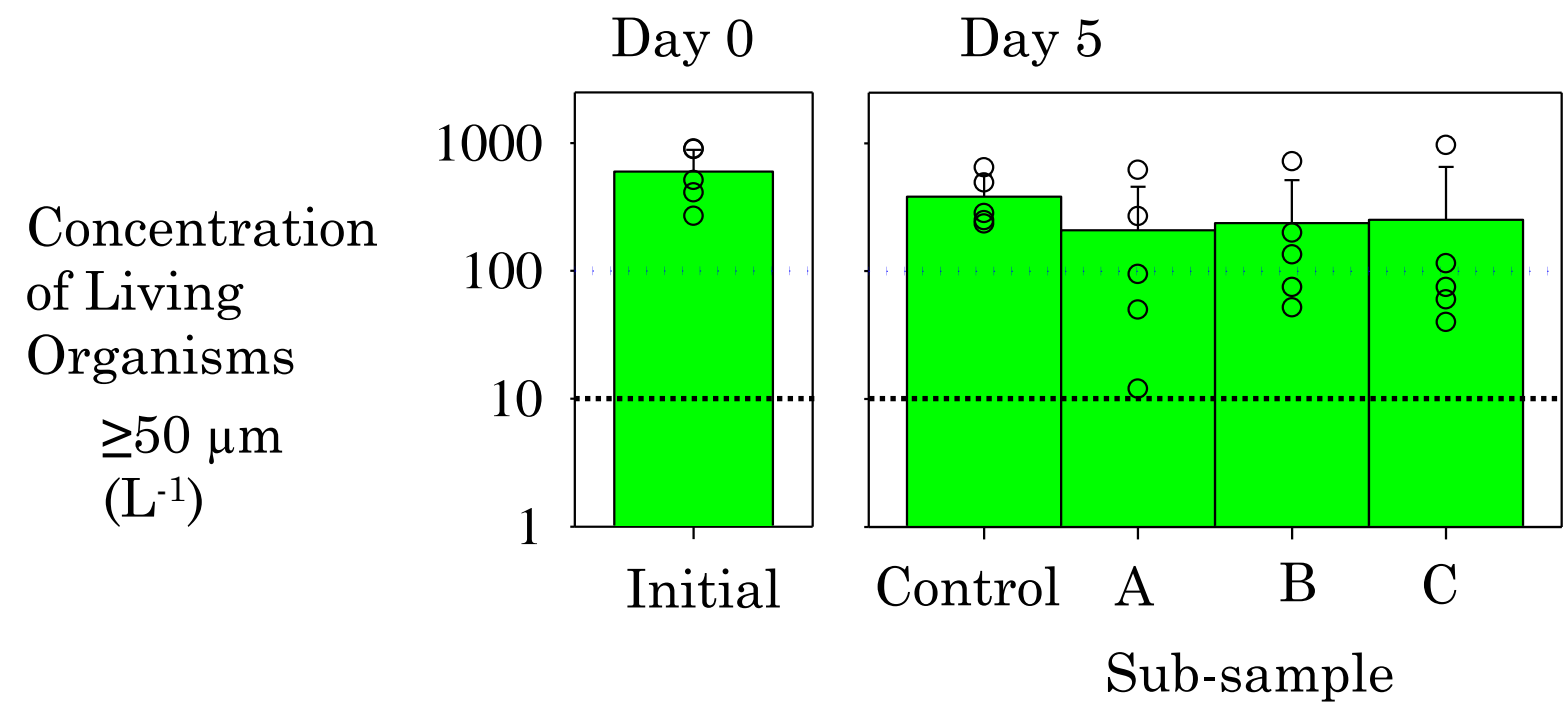
- High variation in F_V/F_M measurements
- Only at 100 mWs cm⁻² were readings sig. diff. from other treatments and only at day 1 (ANOVA, p>0.05)



- Control
- Sig. Diff. from Control
- Sig. Less than Discharge Std.

Bars: mean ± 1 SD
(n = 6; shown as symbols on the bars)

Deoxygenation Results: Organisms $\geq 50 \mu\text{m}$

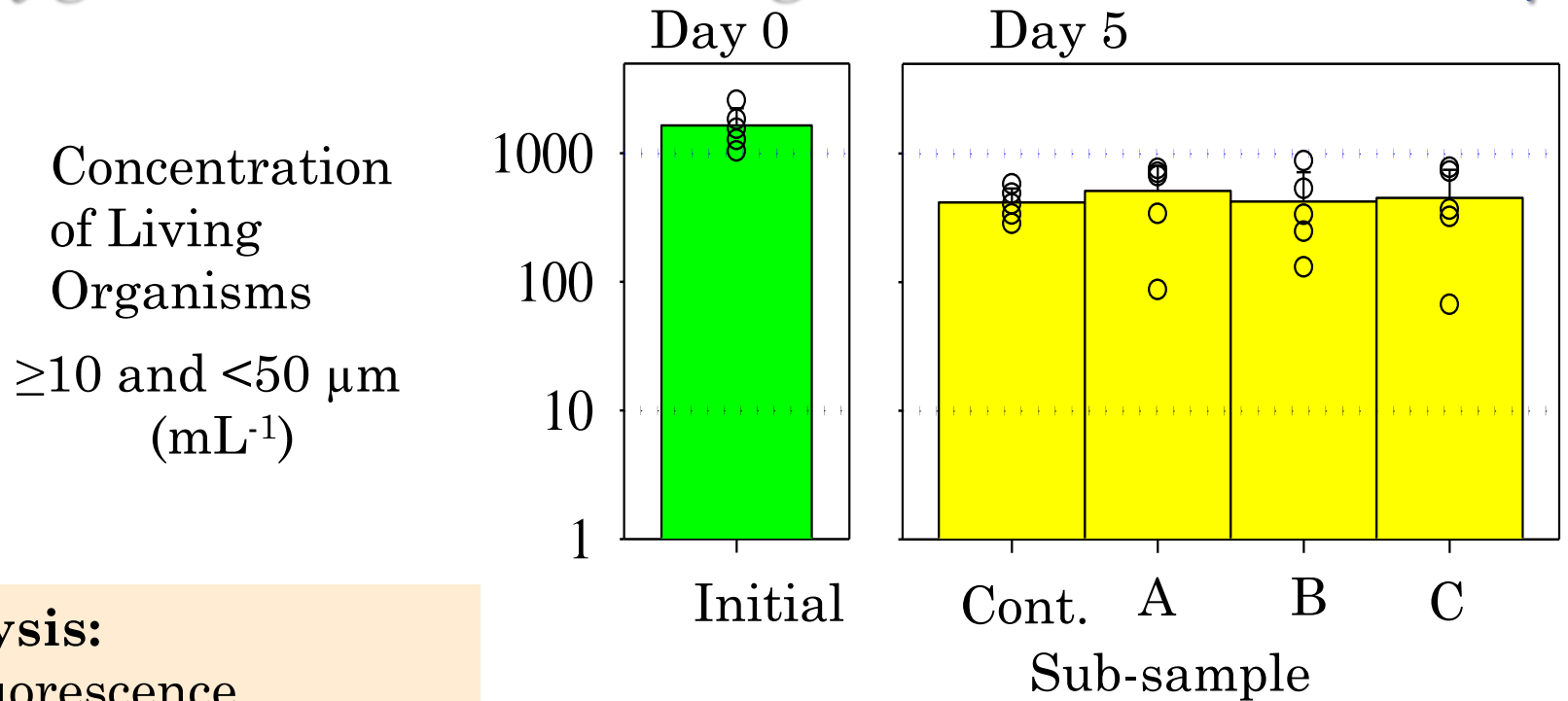


No significant differences in concentrations of organisms $\geq 50 \mu\text{m}$ (ANOVA, $p > 0.05$)

..... ETV Challenge Water
 ----- Discharge Std.
 ■ Control
 ■ Sig. Diff. from Control
 ■ Sig. Less than Discharge Std.

Bars: mean \pm 1 SD
(n = 6; shown as symbols on the bars)

Deoxygenation Results: Organisms ≥ 10 and $< 50 \mu\text{m}$



Analysis:

Epifluorescence microscope counts of organisms labeled with vital stains (Steinberg et al., 2011)

Organisms ≥ 10 and $< 50 \mu\text{m}$ all were sig. diff. from initial (ANOVA, $p < 0.05$)
 But deoxygenated treatments were not sig. diff. from control after day 5 for org. ≥ 10 and $< 50 \mu\text{m}$

Control

Sig. Diff. from Control

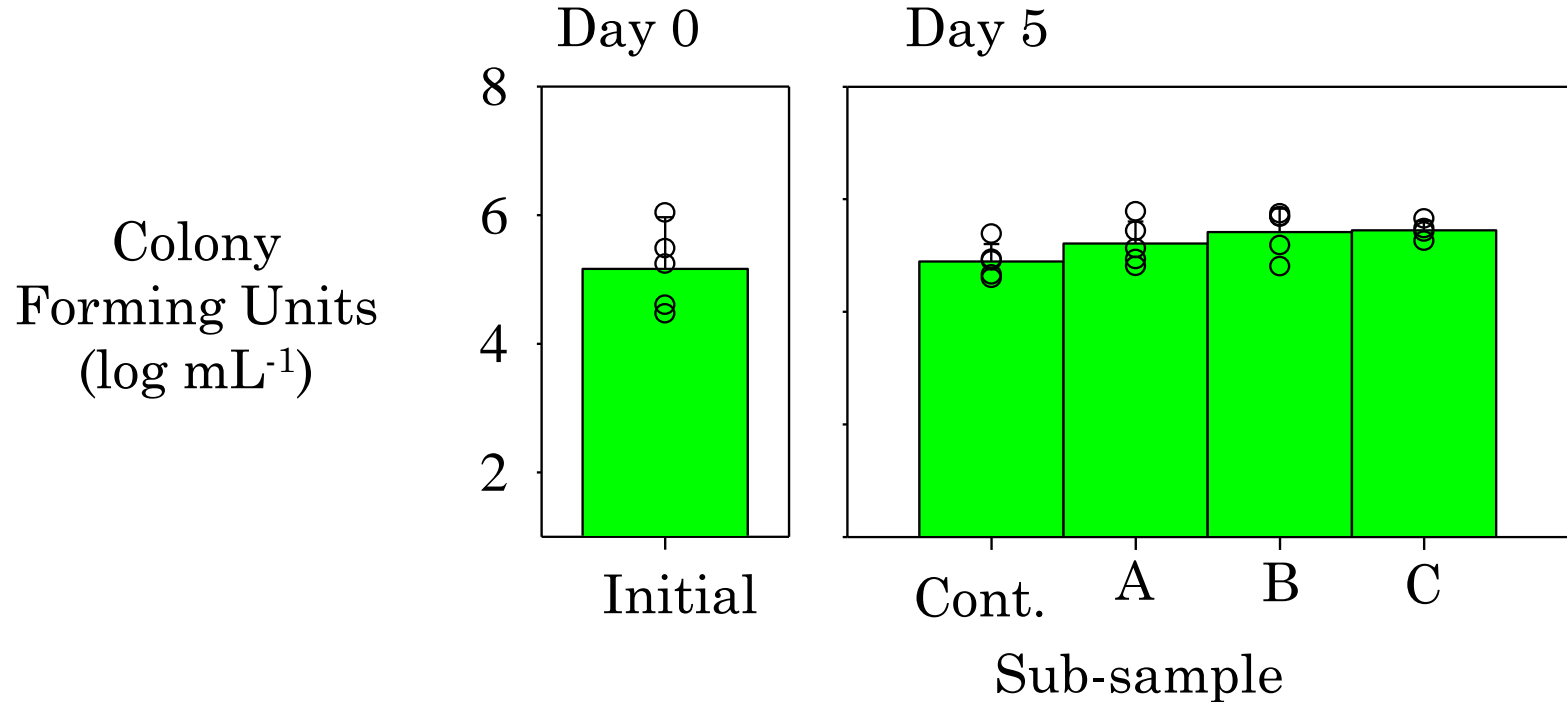
Sig. Less than Discharge Std.

..... ETV Challenge Water Discharge Std.

Bars: mean \pm 1 SD

(n = 6; shown as symbols on the bars)

Deoxygenation Results: Heterotrophic Bacteria



Heterotrophic bacteria:

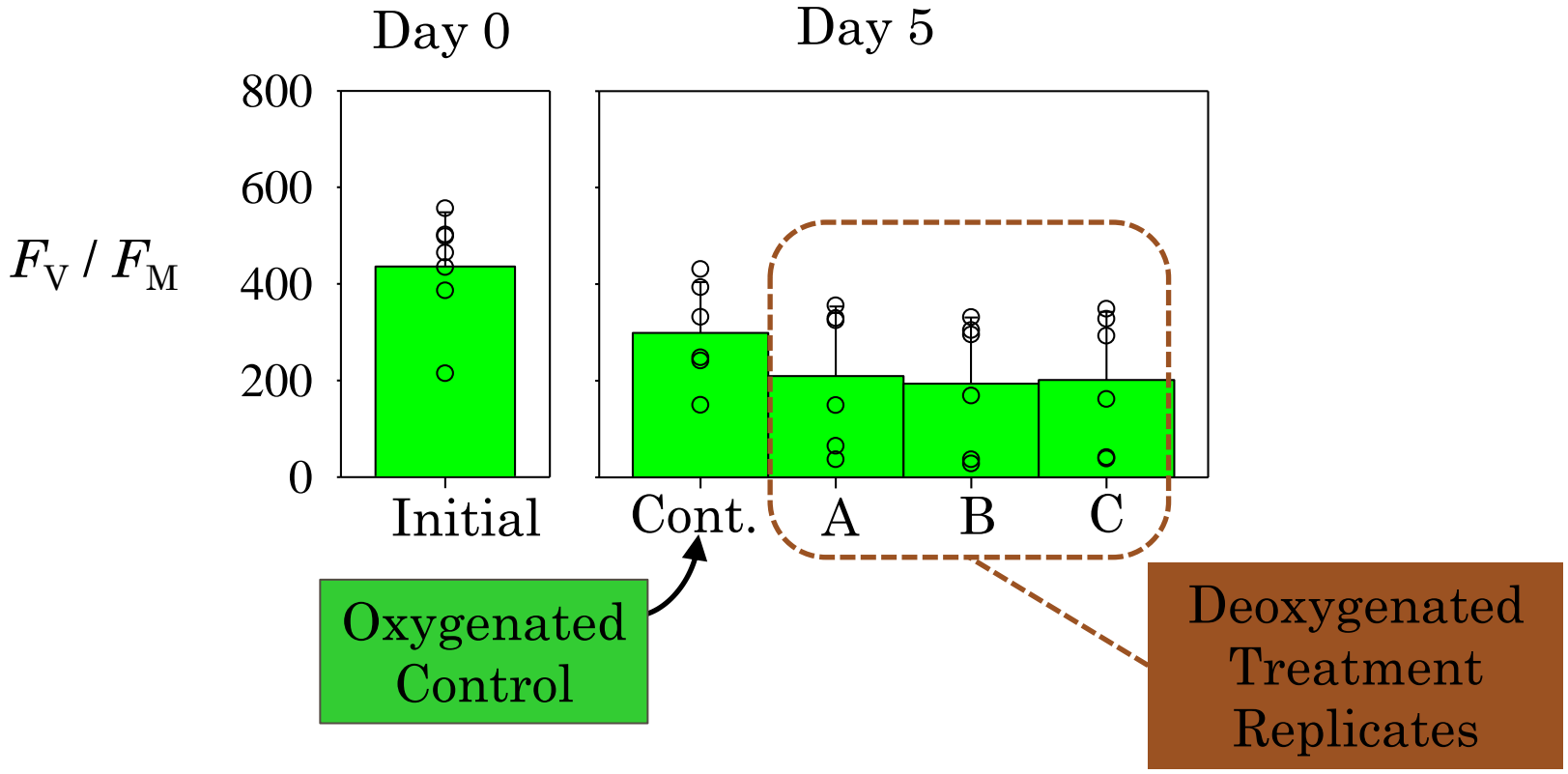
No significant difference in concentrations in controls or treatment replicates (ANOVA, $p > 0.05$)

- Control
- Sig. Diff. from Control
- Sig. Less than Discharge Std.³³

Bars: mean \pm 1 SD
(n = 6; shown as symbols on the bars)

Deoxygenation Results: Variable Fluorescence

F_V / F_M measurements not sig. different from control after day 5 in any of the three replicate treatments (ANOVA, $p > 0.05$)



Bars: mean \pm 1 SD
(n = 6; shown as symbols on the bars)

- Control
- Sig. Diff. from Control
- Sig. Less than Discharge Std.³⁴