Ballast Tank Organisms: Wanted Dead – NOT Alive

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California Prevention First, Sept 10th, 2008

Support from:

U.S. Fish & Wildlife Service

California State Lands Commission

SIMoN Sanctuary Integrated Monitoring Network

- The Problem:
  Abate Aquatic Invasive Species
- The Solution (partial):
  Remove or Inactivate Organisms from Ship Ballast Discharge
- The Challenge:
  Engineer Shipboard Ballast Treatment Systems
- The Situation:
  Scientists must intelligently and accurately distinguish ‘live’ and ‘dead’ organisms in plankton (including microbes) in order to evaluate treatment efficacy and to meet regulations
-

Let’s talk about this…

Table 1. Example regulatory standards for ballast water.

	Mesoplankton	Nanoplankton	Bacteria, Viruses
International Convention for the Control and Management of Ships Ballast Water & Sediments; IMO 2004	**< than 10 viable organisms per m3 for those organisms > 50 µm minimum dimension**	**< 10 viable organisms per mL for those organisms > 10 µm but < 50 µm minimum dimension**	**< 10 CFU per L of *Vibrio cholorae* < 1000 CFU per L of intestinal enterococci < 2500 CFU per L of *Escherichia coli***
Ballast Water Management Act of 2005; US Senate Bill 363, February 10th, 2005	**< than 0.1 viable organisms per m3 for those organisms > 50 µm minimum dimension**	**< 0.1 viable organisms per mL for those organisms > 10 µm but < 50 µm minimum dimension**	**< 10 CFU per L of *Vibrio cholorae* < 330 CFU per L of intestinal enterococci < 1260CFU per L of *Escherichia coli***
Report and Recommendation of the California Advisory Panel on Ballast Water Performance Standards (Staff recommendations, November 2005)	**No detectable viable organisms > 50 µm minimum dimension**	**< 10 viable organisms per L for those organisms > 10 µm but < 50 µm minimum dimension**	**< 10 CFU of bacteria per mL < 100 viruses per mL < 10 CFU per L of *Vibrio cholorae* < 330 CFU per L of intestinal enterococci < 1260 CFU per L of *Escherichia coli***

Plankton Size Distribution

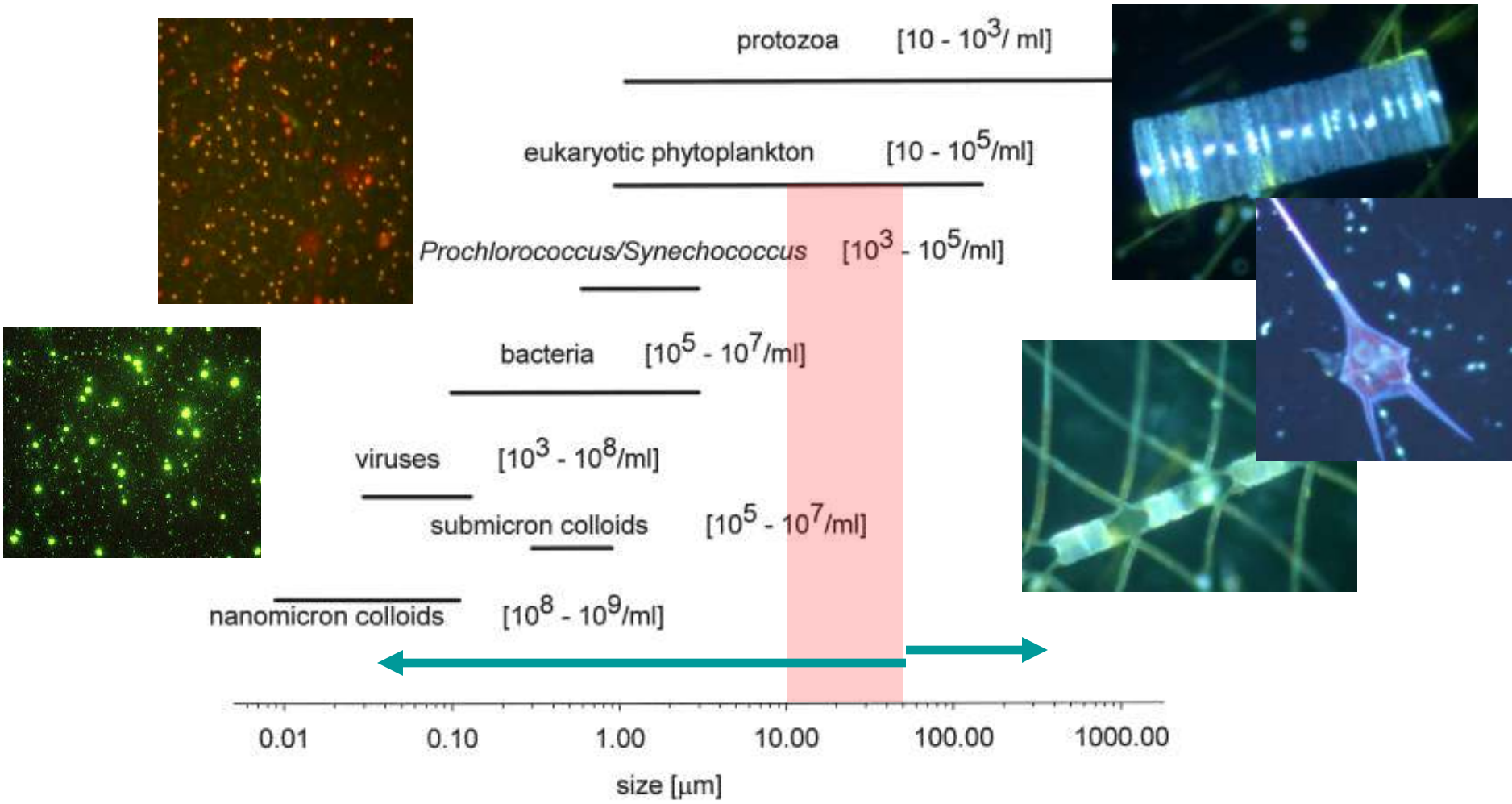


FIG. 1. – Abundances and size class distribution of living and non-living particles in the upper ocean (modified after Koike *et al.*, 1990).(from Veldhuis & Kraay 2000)

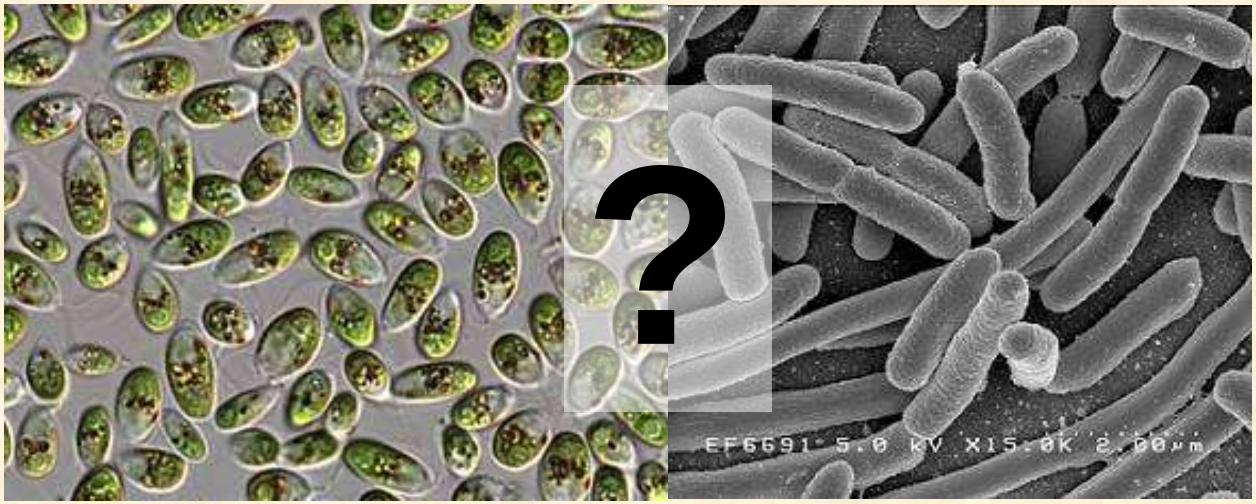
Dead or Alive?

Dead

Alive

Dead

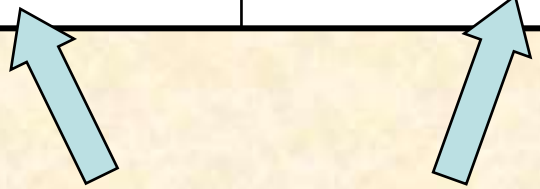
Alive



Definition of unicellular viability

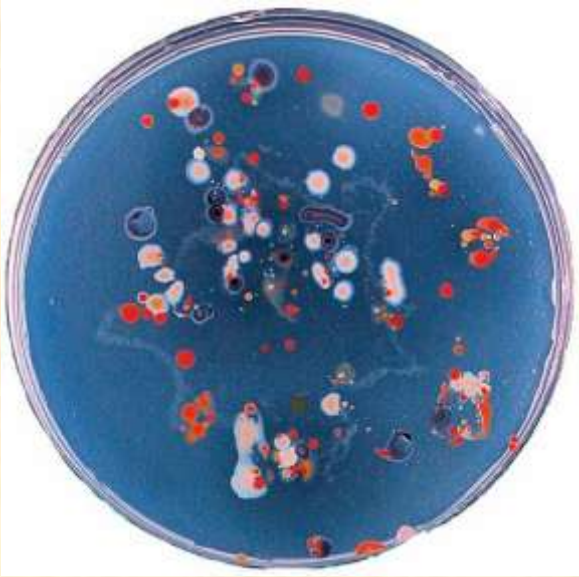
- Cells capable of growth (cell division) and metabolism

	Capable of growth	Metabolically active
Viable	X	X
Viable but inactive Dormant	X	-
Active – **non cultivable**	?	X
Dead	-	-

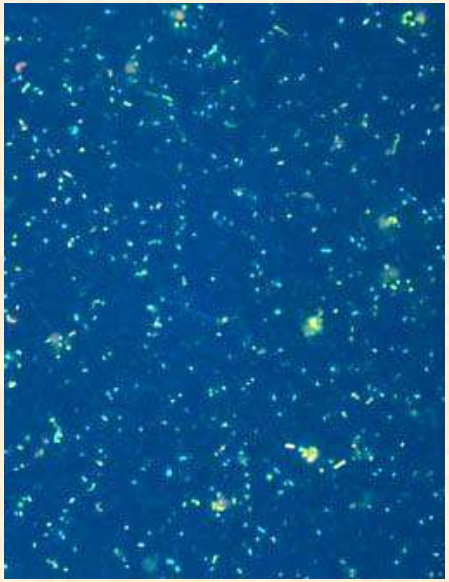


To score a cell as truly live or dead, we must measure growth capacity and metabolic activity

Colony forming units vs. direct count



www.niwa.cri.nz



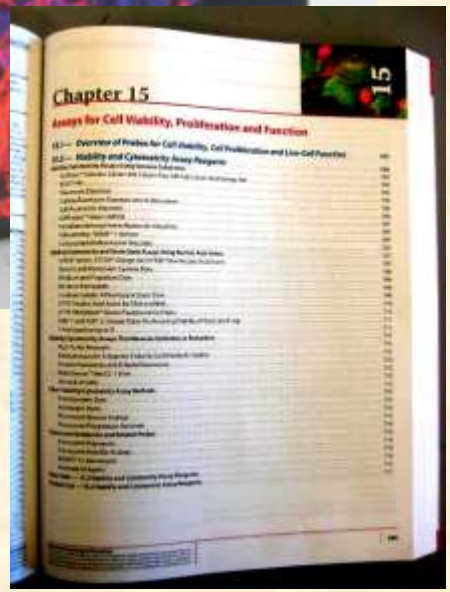
www.dees.dri.edu

(Daley & Hobbie 1975)

0.1-10% of direct count

- 90 – 99.9% of bacterial cells are…
  - Dead?
  - Dormant/Inactive?
  - Non-cultivable?

The handbook



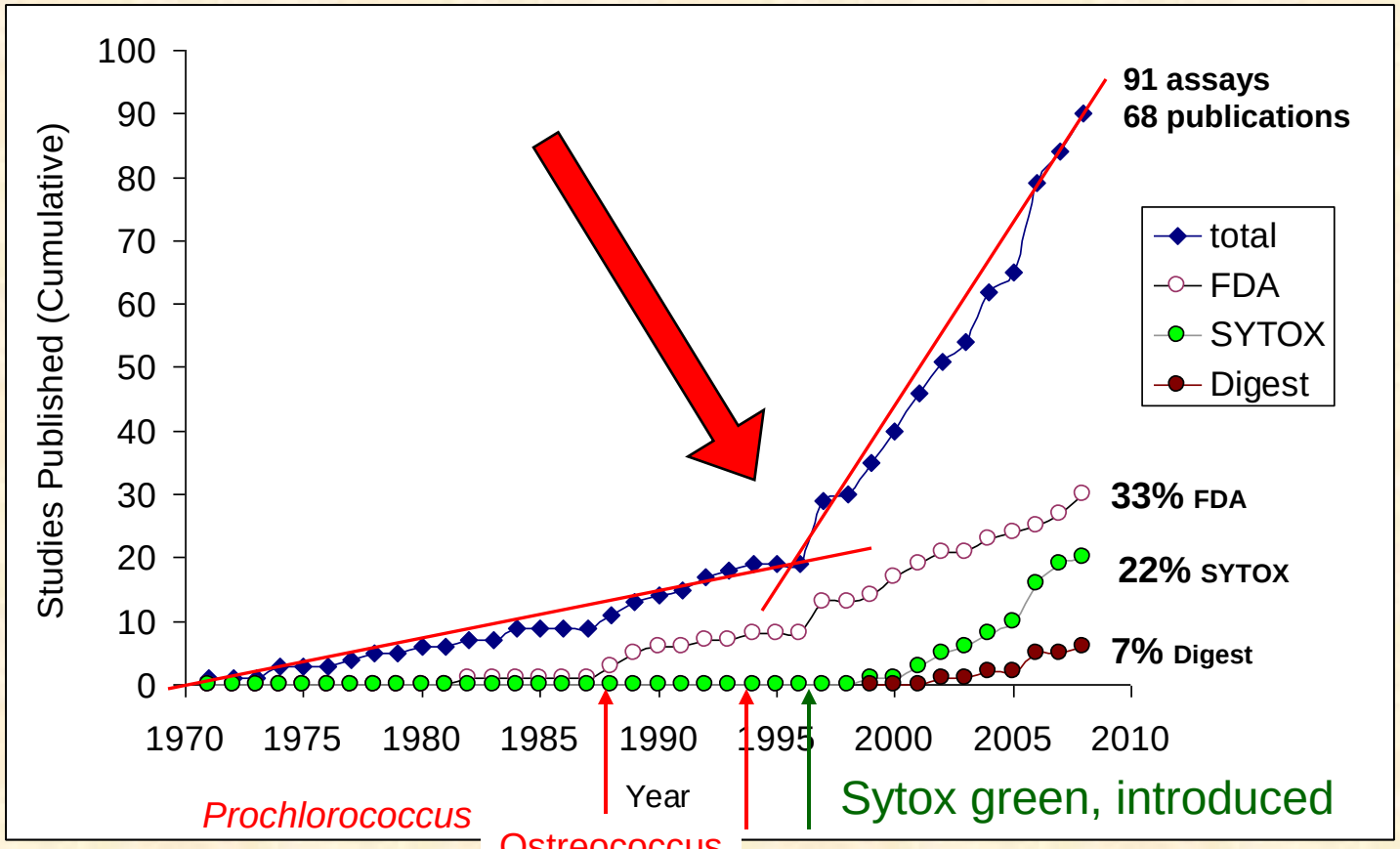
Chapter 15: Assays for Cell Viability, Proliferation and Function

- **All** of these stains are designed to be used in human health related research
  - Mammalian cell lines
  - *E. coli*, other pathogens
- **None** of these stains is designed for or optimized for environmental studies
- Many not suitable for use with phytoplankton
  - Autofluorescence interference
  - Impaired staining under variable pH, temp, etc.

Selected Vital Stains

Stain/Dye	Assay target	Result
CTC	cell respiration	Live cells fluoresce red
Calcein AM	intracellular esterase activity	Live cells fluoresce green
BCECF AM	intracellular esterase activity	Live cells fluoresce green
FDA	intracellular esterase activity	Live cells fluoresce green
CFDA-AM	intracellular esterase activity	Live cells fluoresce green
CDFA	intracellular esterase activity	Live cells fluoresce green
trypan blue	Membrane Integrity	Dead cells stain blue
Evan's Blue	Membrane Integrity	Dead cells stain blue
SYTOX® Green	Membrane Integrity	DNA of dead cells fluoresce green
7-AAD	Membrane integrity	DNA of dead cells fluoresce red
EthD-1	Membrane Integrity	DNA of dead cells fluoresce red-orange
PI	Membrane Integrity	DNA of dead cells fluoresce red-orange
DiOC2	mitochondrial membrane potential	Mitochondria of live cells fluoresce red
TTC	redox potential	medium fluoresces red with live cells
XTT	redox potential	medium turns orange
MTT	redox potential	medium turns purple
resazurin	Unid. intracellular enzymatic/chemical activity	medium fluoresces pink in presence of live cells
alamarBlue™	Unid. intracellular enzymatic/chemical activity	medium fluoresces pink in presence of live cells

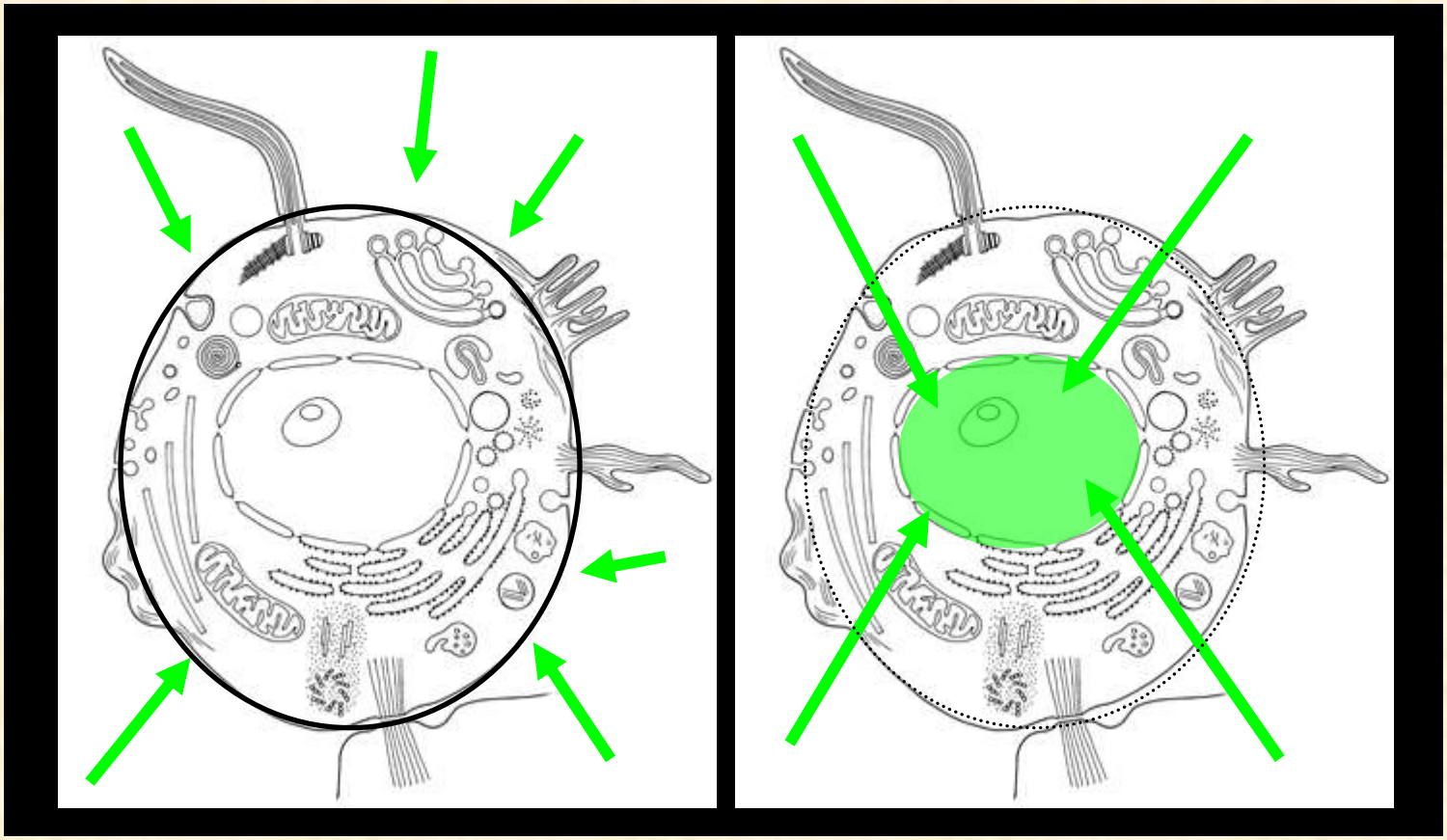
Published phytoplankton viability studies



Selected phytoplankton viability studies published from 1971 to present . Data are cumulative and represent viability studies from a significant, though not comprehensive, survey of the literature.

(Watt 1971, Crippen & Perrier 1974, Faust & Correll 1977, Paerl 1978, Reynolds et al. 1978, Descolas-Gros 1980, Bentley-Mowat 1982, Gallagher 1984, Berglund & Eversman 1988, Yentsch et al. 1988, Dorsey et al. 1989, Selvin et al. 1989, Gala & Giesy 1990, Berdalet & Dortch 1991, Gilbert et al. 1992, Arsenault et al. 1993, Minier et al. 1993, Gala & Giesy 1994, Reiriz et al. 1994, Faber et al. 1997, Geary et al. 1997, Lee & Rhee 1997, Murphy & Cowles 1997, Pouneva 1997, Regel 1997, Berges & Falkowski 1998, Jochem 1999, Lee & Rhee 1999, Okochi et al. 1999, Vardi et al. 1999, Brookes et al. 2000, Onji et al. 2000, Vasconcelos et al. 2000, Brookes et al. 2001, Brussaard et al. 2001, Franklin et al. 2001, Veldhuis et al. 2001, Agustí & Sanchéz 2002, Regel et al. 2002, Anderson et al. 2003, Segovia et al. 2003, Agustí 2004, Berman-Frank et al. 2004, Franklin & Berges 2004, Franklin et al. 2004, Latour et al. 2004, Regel et al. 2004, Casotti et al. 2005, van de Poll et al. 2005, Agustí et al. 2006, Binet & Stauber 2006, Franklin et al. 2006, Gregg & Hallegraeff 2006, Lawrence et al. 2006, Llabrés & Agustí 2006, Moharikar et al. 2006, van de Poll et al. 2006, Vardi et al. 2006, Garvey et al. 2007, Jansen & Bathmann 2007, Ribalet et al. 2007, Timmermans et al. 2007, Wigglesworth-Cooksey et al. 2007, Hayakawa et al. 2008, Holm et al. 2008, Prince et al. 2008)

SYTOX Green (marks ‘dead’ cells)



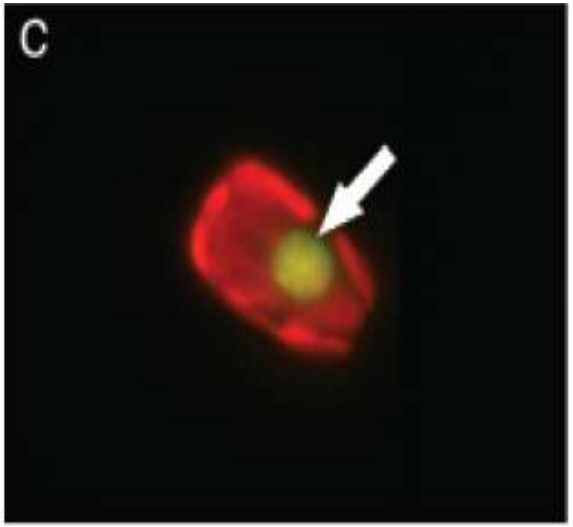
Intact Membrane – no stain

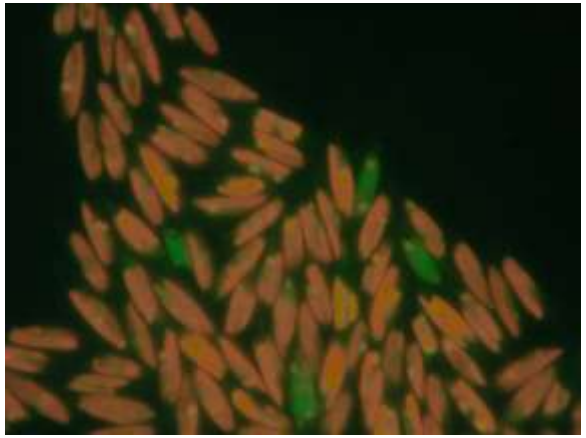
LIVE

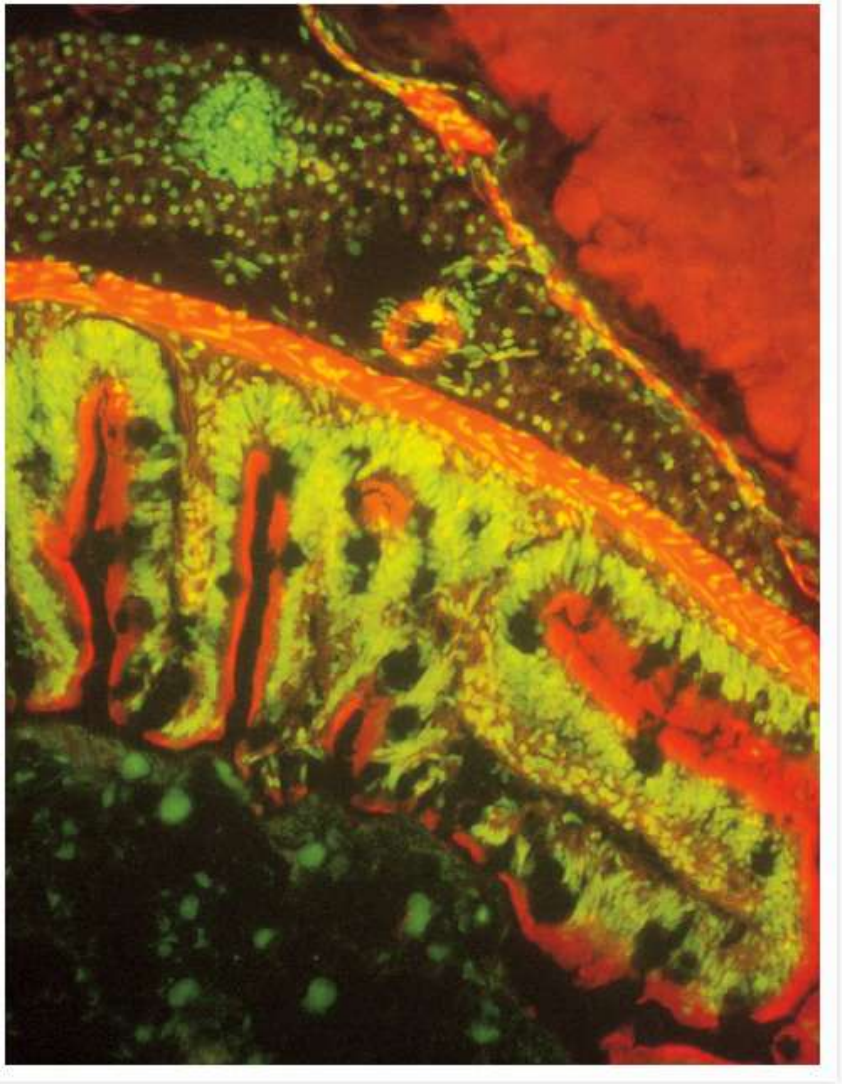
Permeable membrane – stained nucleus

DEAD

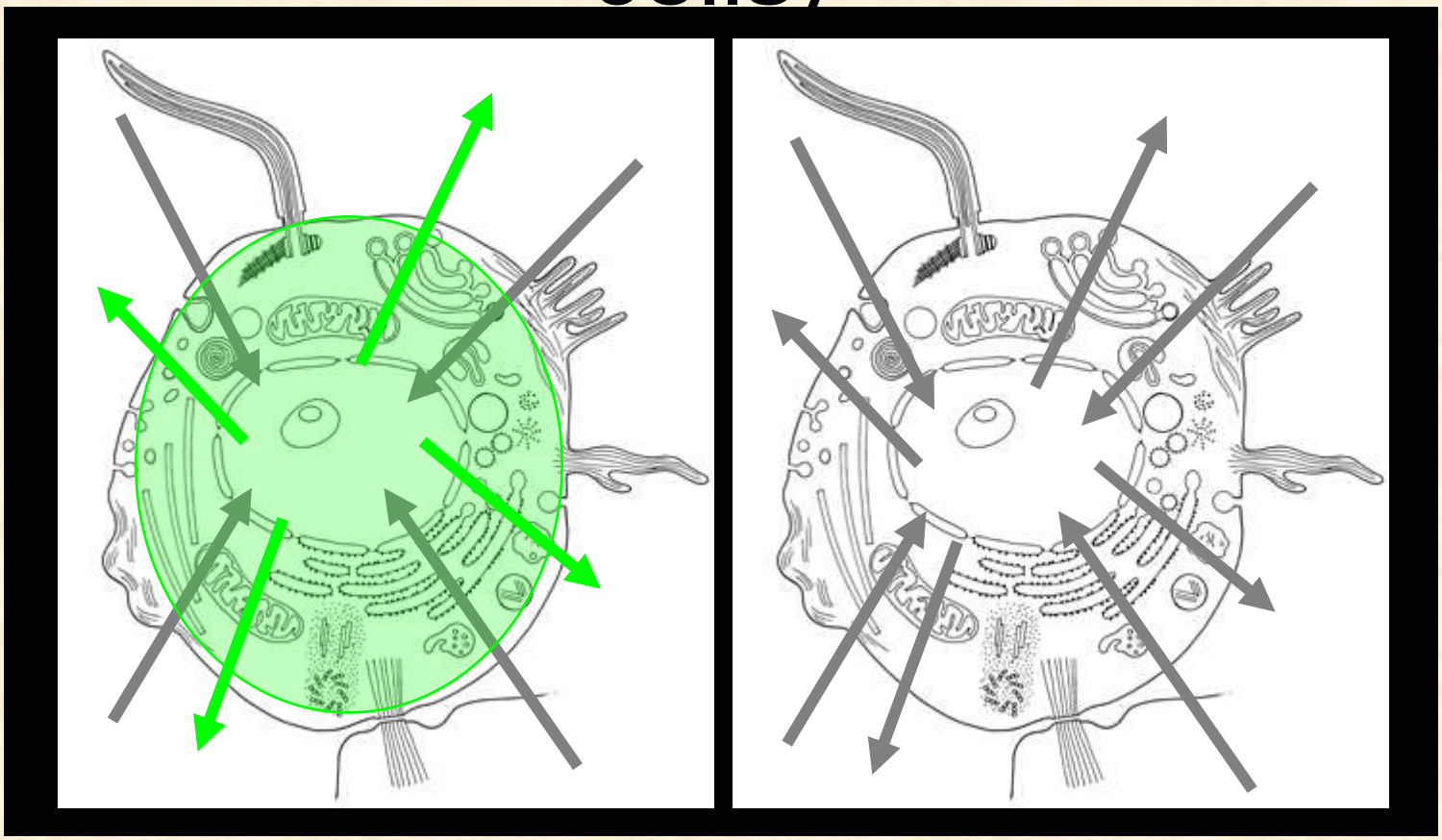
SYTOX® Green Visible Fluorescence







Fluorescein Diacetate (marks ‘live’ cells)



Enzyme activity – stained

LIVE

No enzyme activity – unstained

DEAD

Fluorescein Diacetate
visible Fluorescence

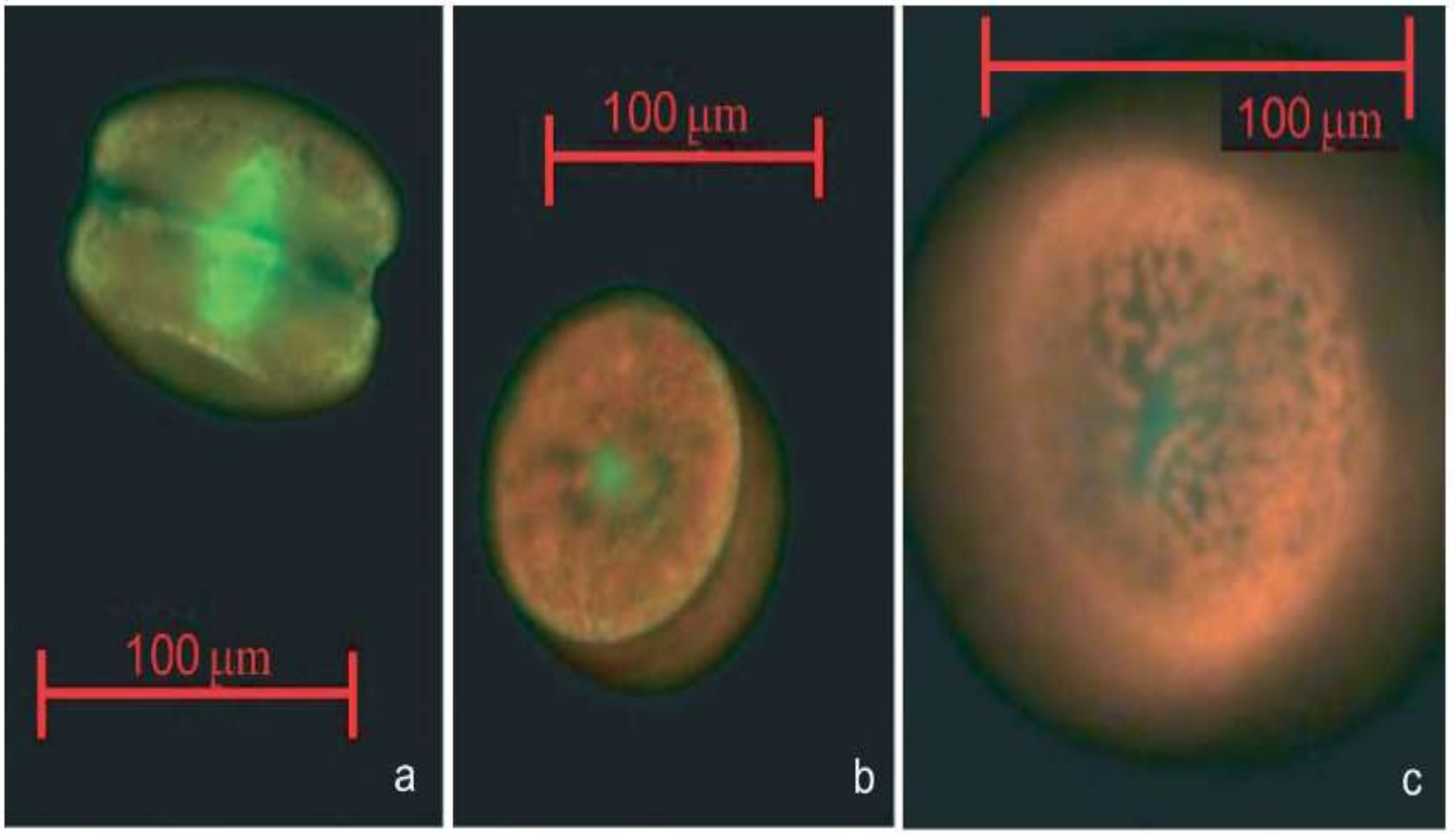
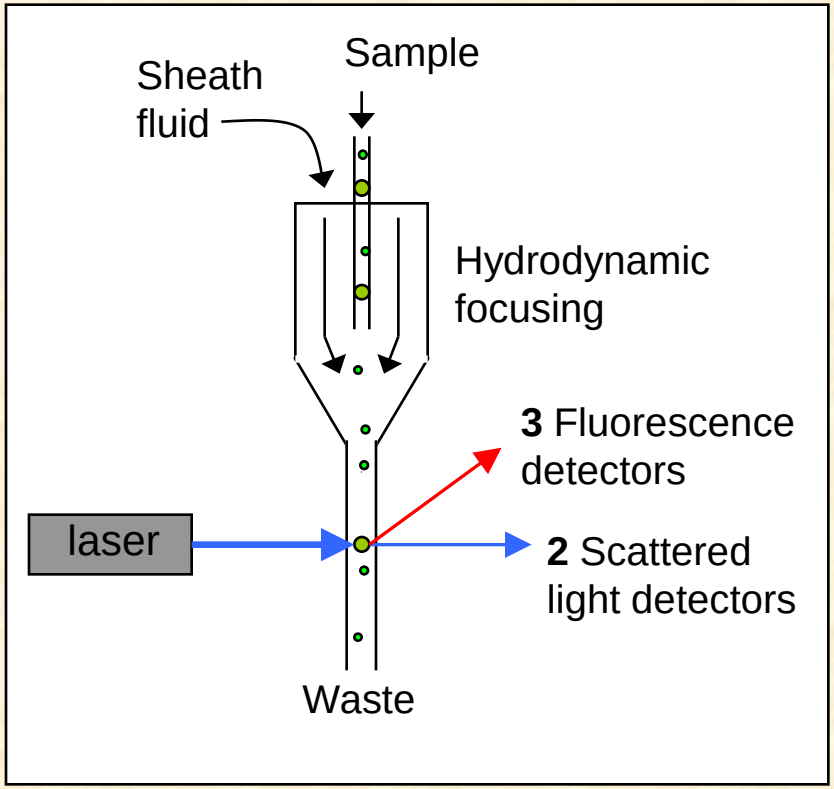


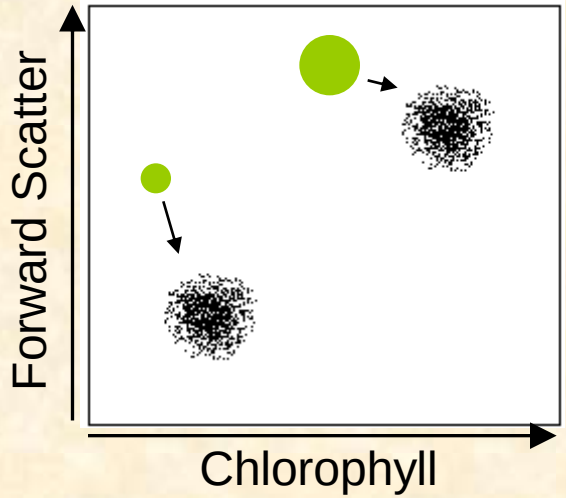
Fig. 1. *Coscinodiscus granii*. Photographs of *C. granii* stained with fluorescein diacetate (FDA) and viewed under blue light. The red autofluorescence is so strong that, depending on the angle at which the cell is observed, the green FDA fluorescence can be obscured. (a) Green fluorescence is clear from the side view, but (b) hidden from the top view, and (c) disappears under exposure to white light

Garvey et al. 2007

Anatomy of a Flow Cytometer



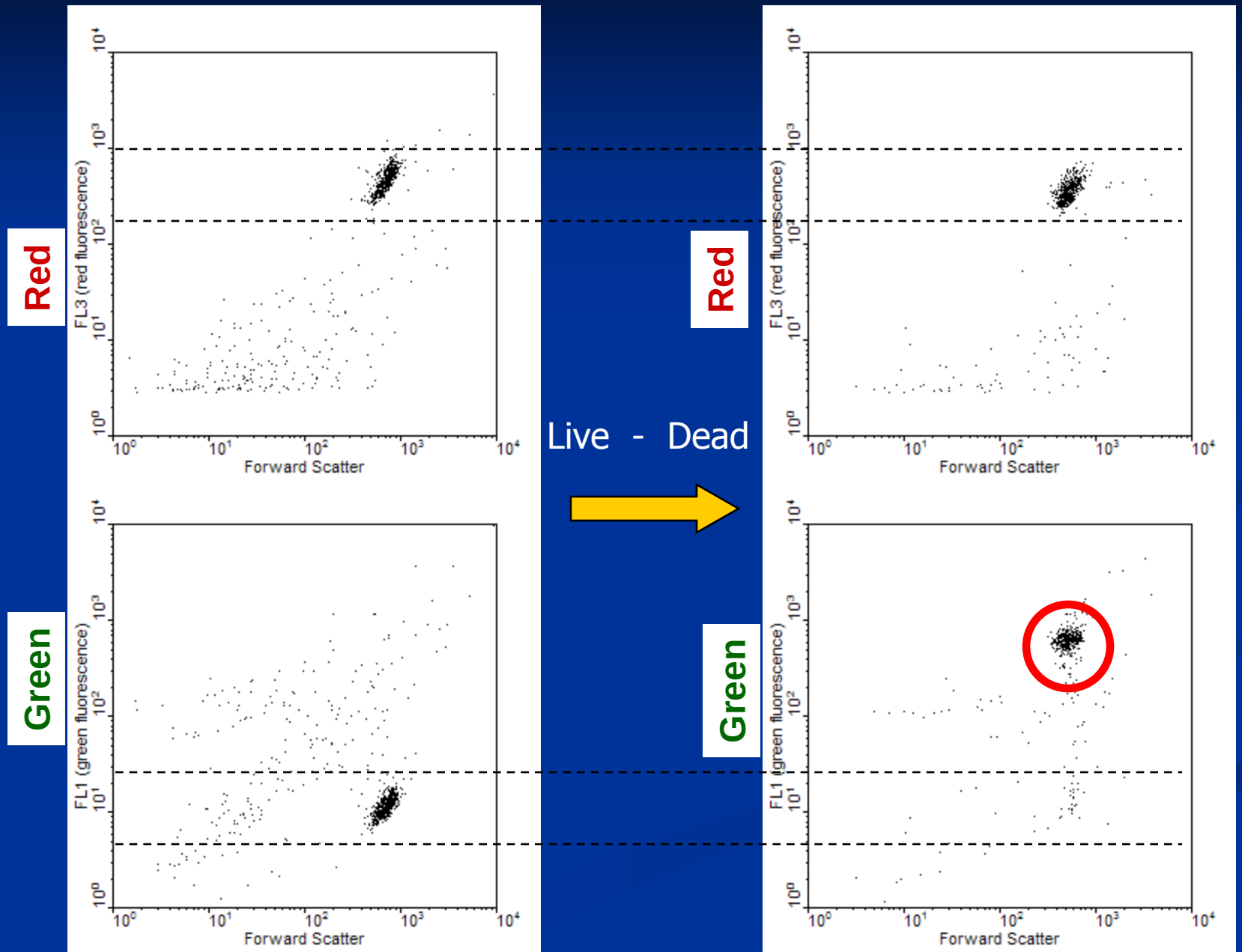
Detector	*Measures*
Forward Scatter	**Size**
Side Scatter	**Shape**
Red Fluorescence	**Chlorophyll autofluorescence**
Orange Fluorescence	**Phycobilin autofluorescence**
Green Fluorescence	**Stains** *mild autofluorescence*



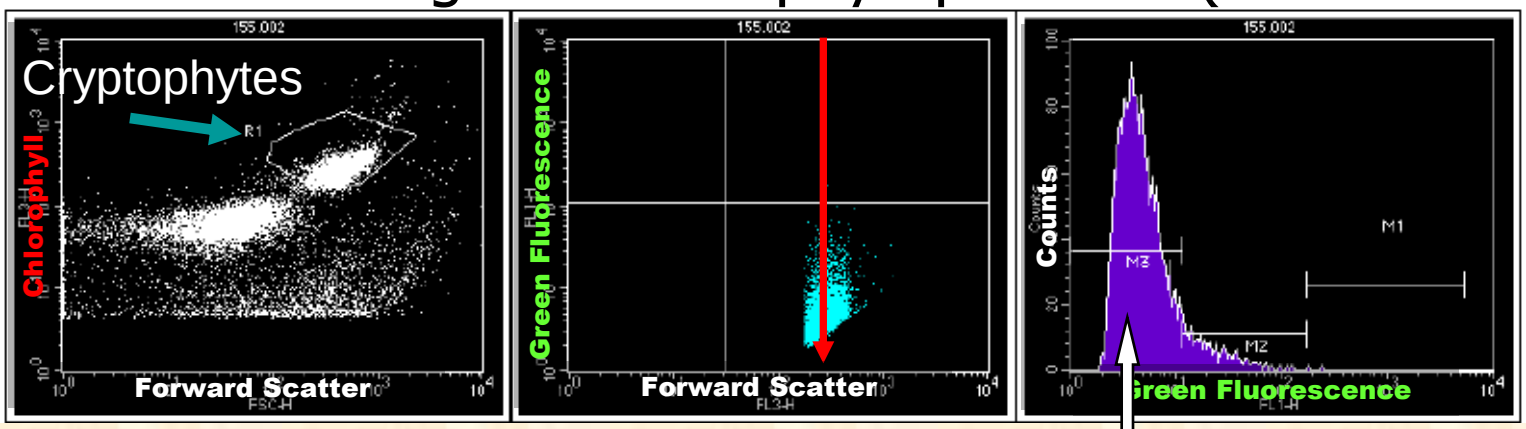
Flow Cytometric Analysis of Viability – Sytox Green

Live Phaeodactylum

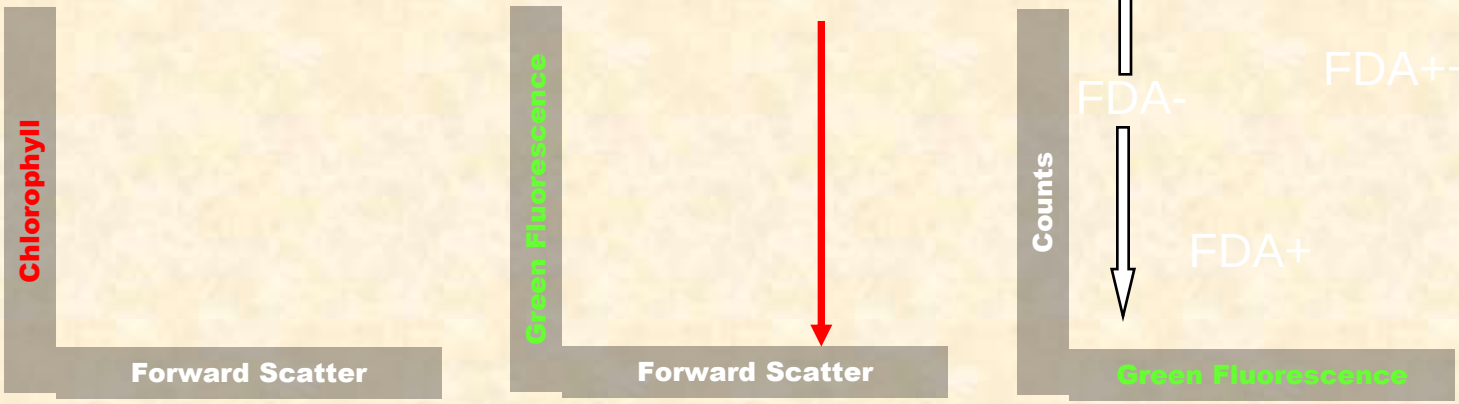
Dead Phaeodactylum (Glutaraldehyde)

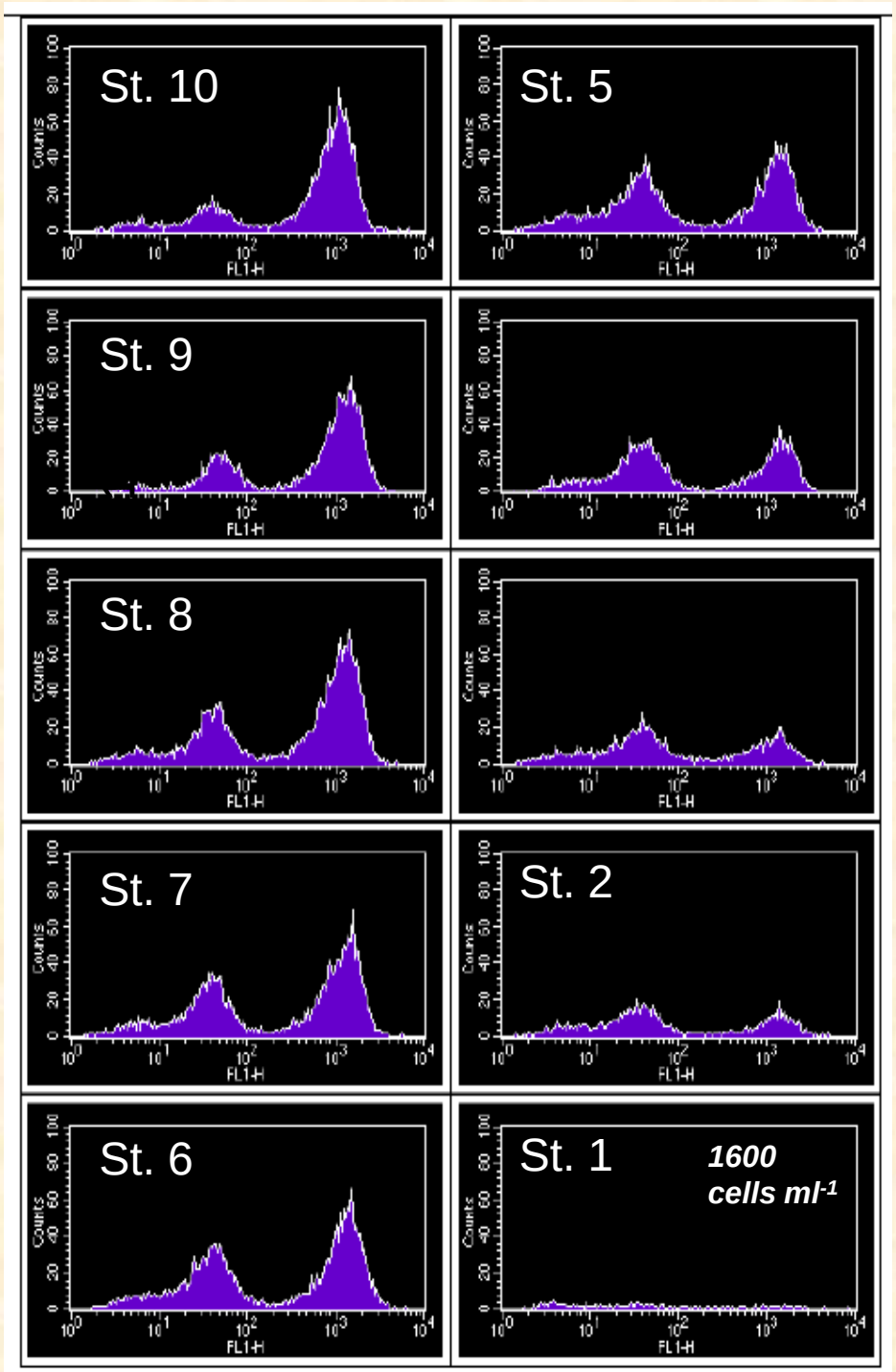


FDA staining of natural phytoplankton (Elkhorn Slough)

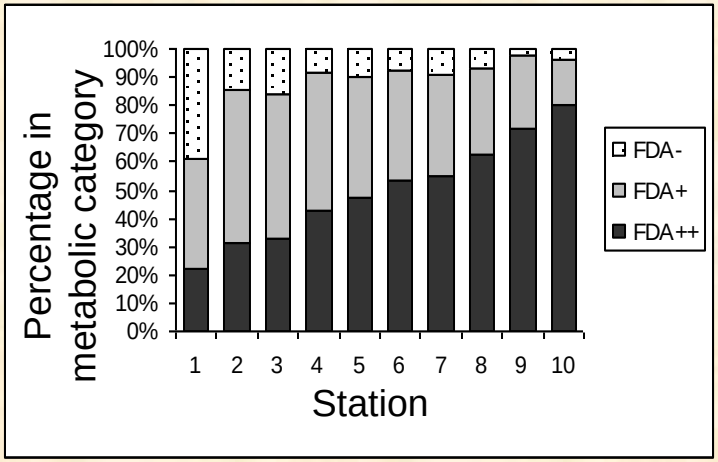


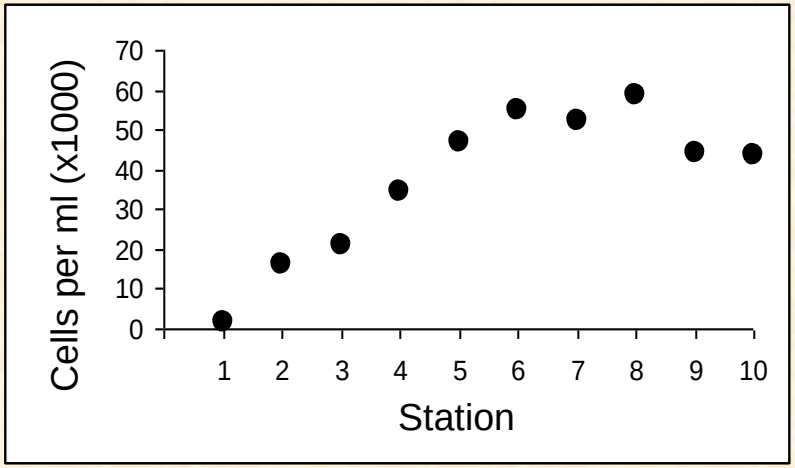
Unstained



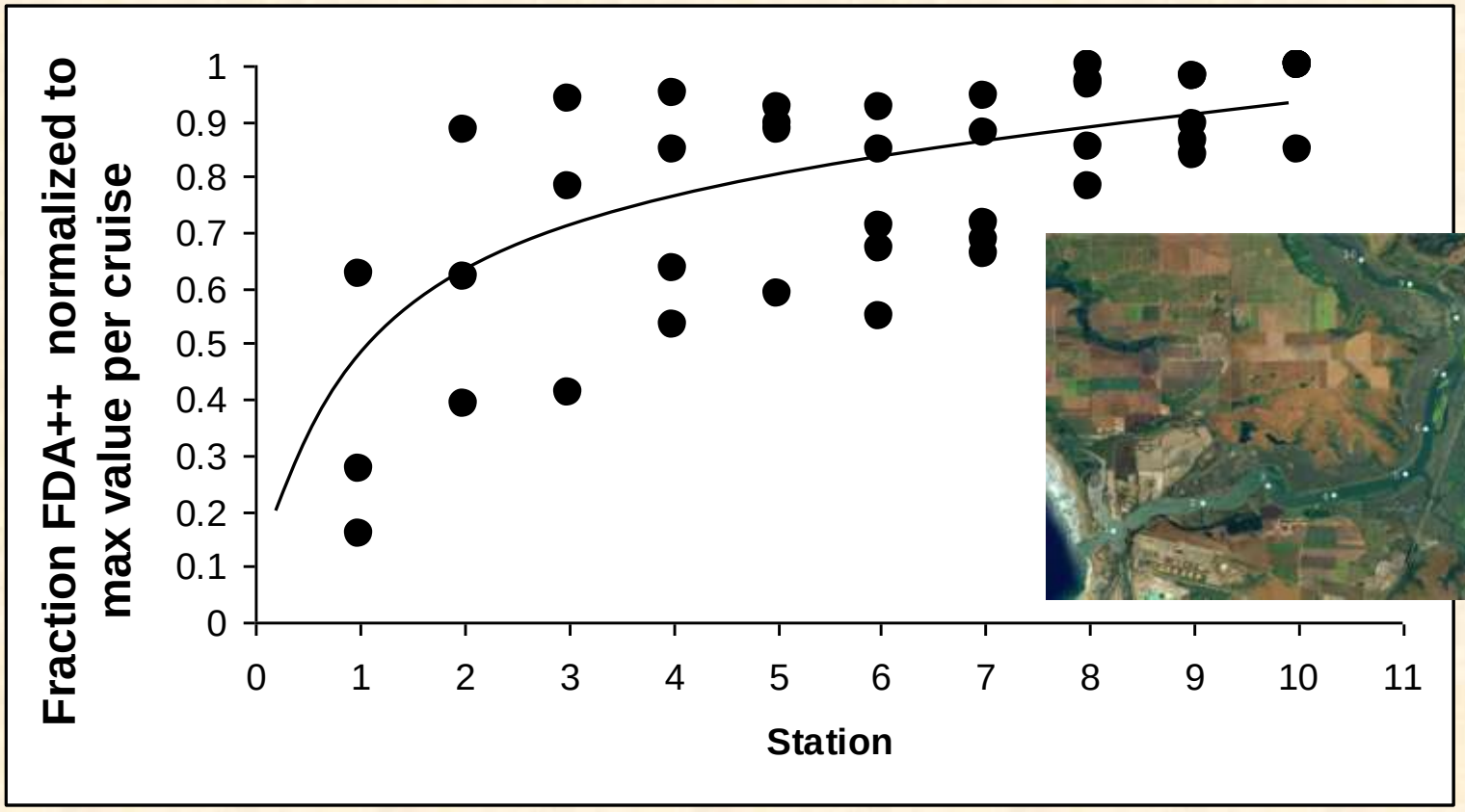


FDA Staining Results: Continued





Viability Summary



Small cryptophytes (~3µm) show an increased fraction of metabolically active cells in the upper Elkhorn Slough

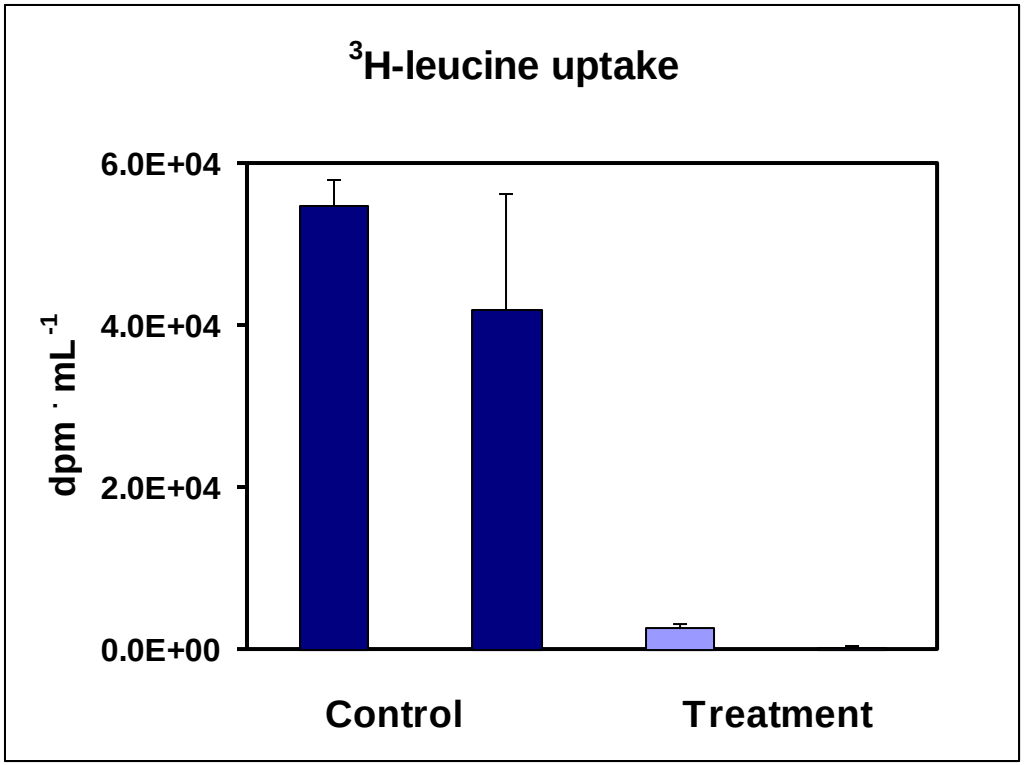
Analysis of viability stains is time-consuming, costly and impractical at times

Cell-specific viability analysis is not the only show in town…

What about physiological metabolism?

Examples of ‘bulk’ metabolic activity in ballast-related experiments

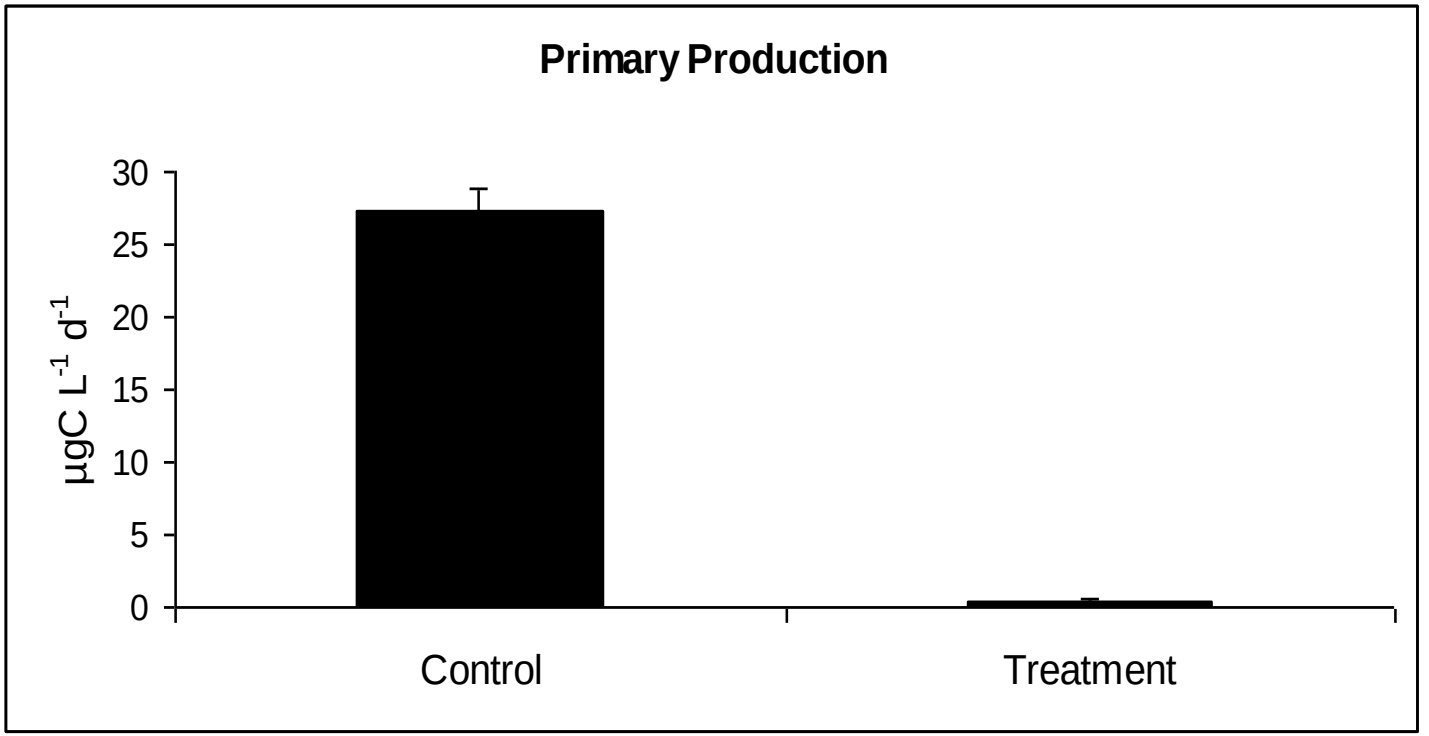
Bacterial Heterotrophic Production



5 days after treatment with chlorine dioxide (5 ppm)
M/V *Atlantic Compass*, February 2007
Smith, Cox and Maranda (unpublished)

Examples of ‘bulk’ metabolic activity in ballast-related experiments

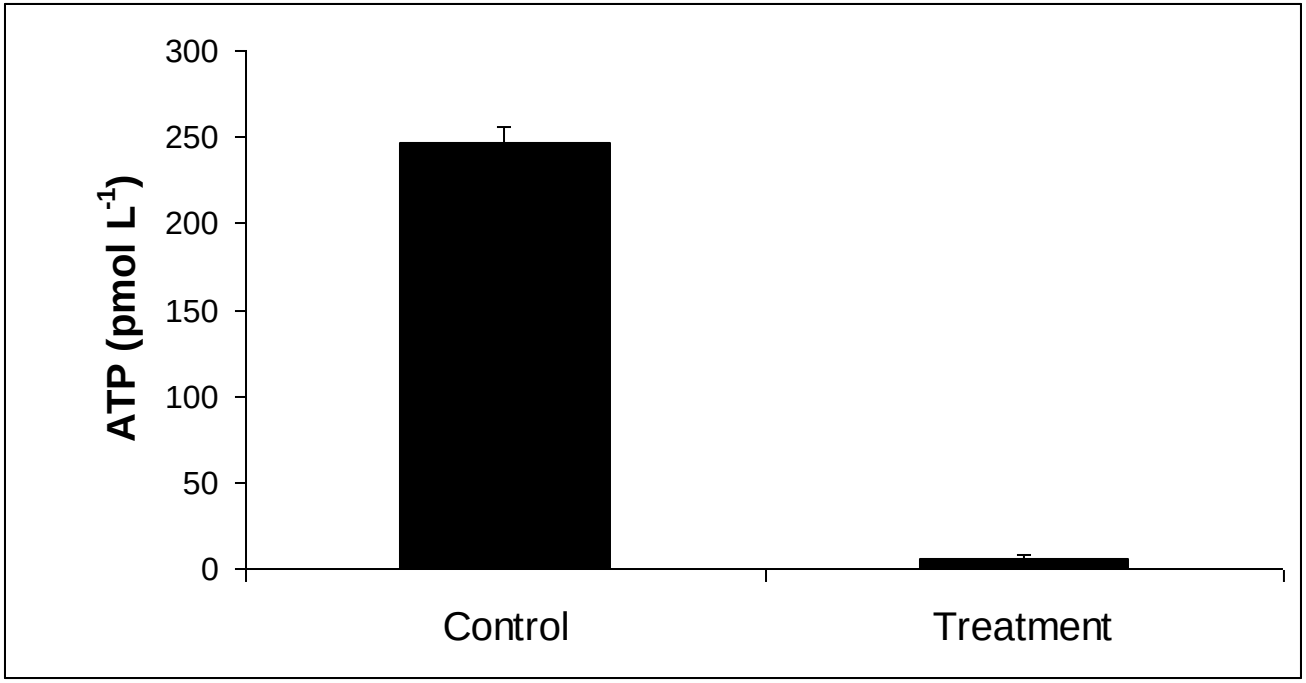
Phytoplankton
Photosynthesis
C-14 technique



Photosynthesis (^{14}C) Ballast Treatment Expt.
M/V *Eversuperb*, MARENCO UV/filter treatment
Welschmeyer et al 2007

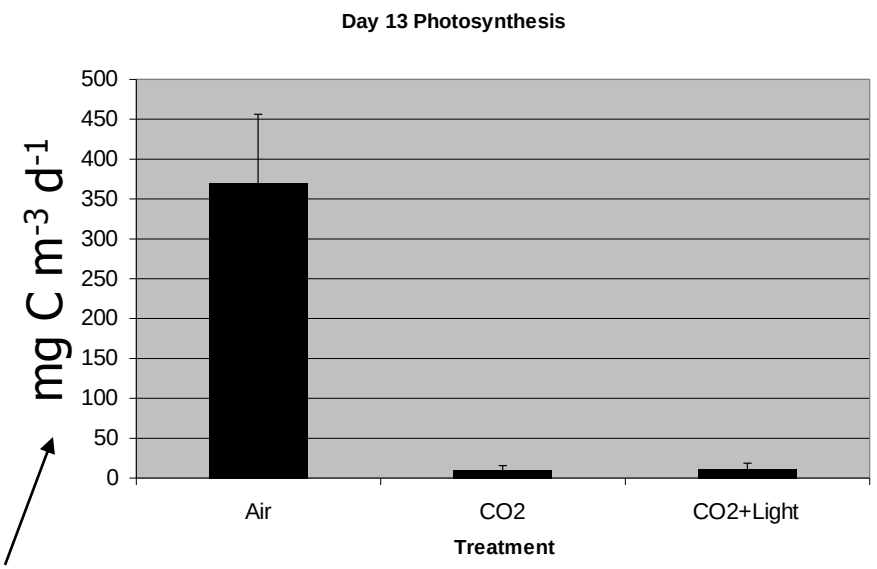
Examples of ‘bulk’ metabolic proxies in ballast-related experiments

Microbial (<50um)
ATP content



ATP concentration during Ballast Treatment Expt.
M/V *Eversuperb*, MARENCO UV/filter treatment
Welschmeyer et al 2007

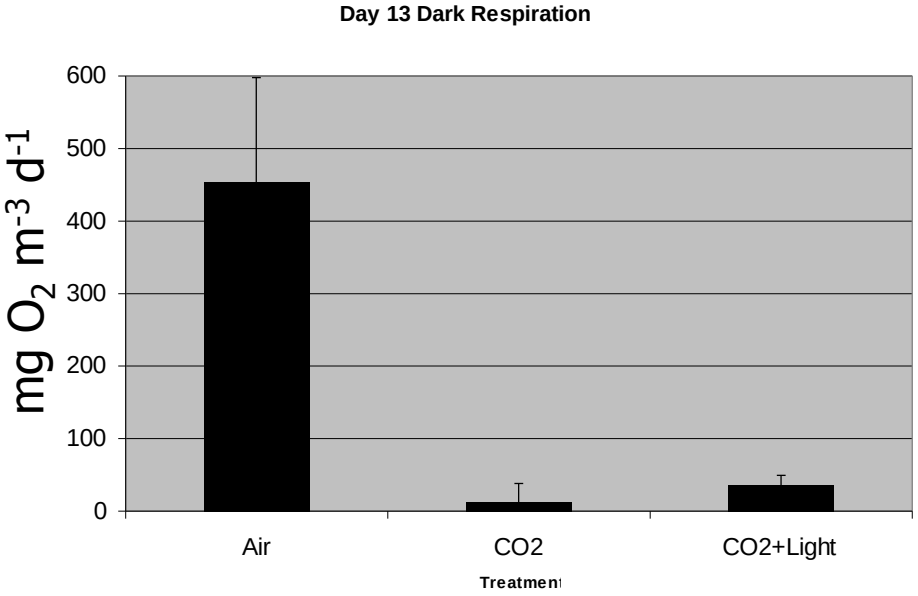
Examples of ‘bulk’ metabolic activity in ballast-related experiments



Photosynthetic C-14 uptake

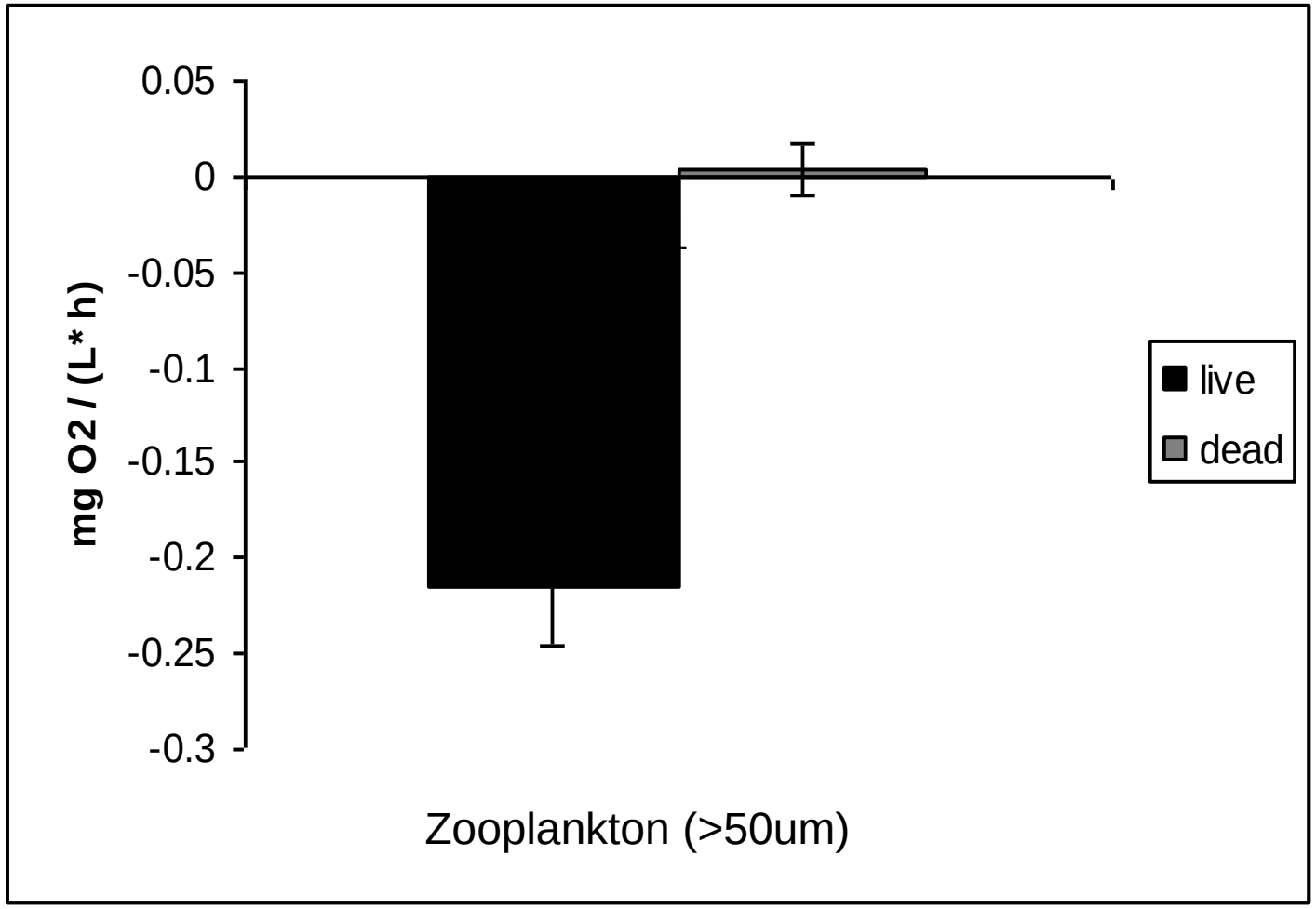
Rau et al (in prep). CO_2 as a biocide; 7 days after 25% CO treatment ended.

Microbial Dark Oxygen Respiration (Winkler)



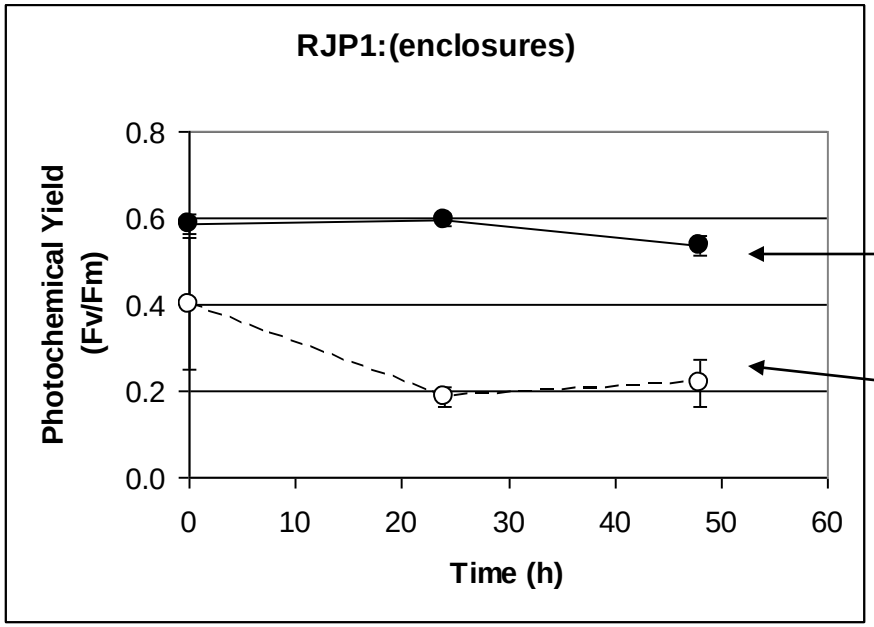
Examples of ‘bulk’ metabolic proxies in ballast-related experiments

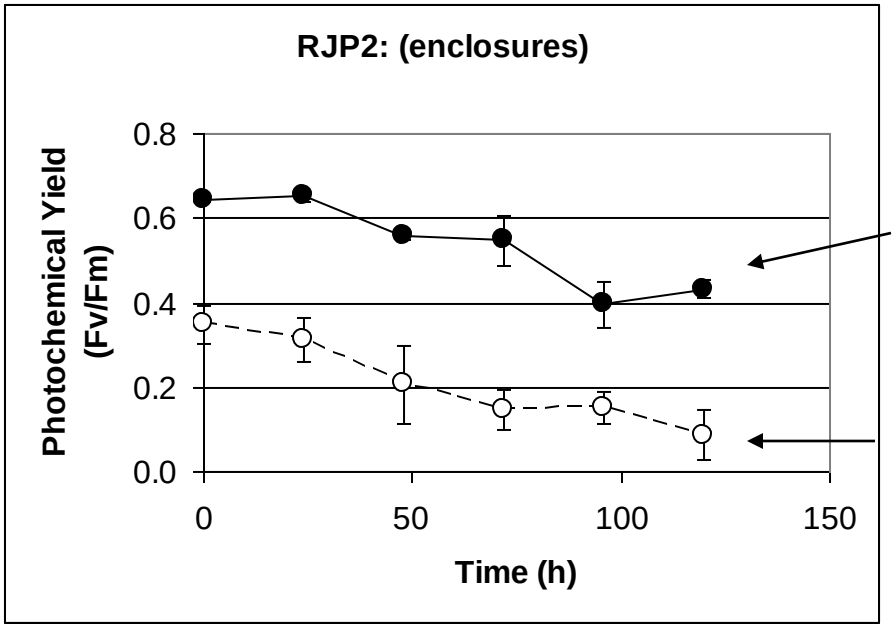
Zooplankton
Respiration
(oxygen electrode)
1% Sodium Azide



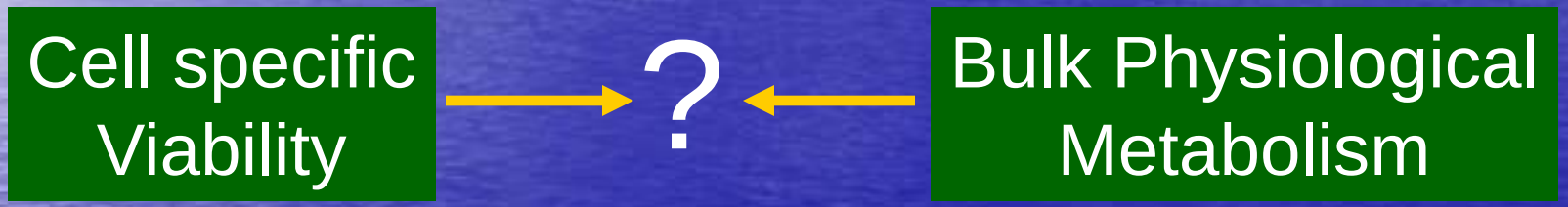
Examples of ‘bulk’ metabolic proxies in ballast-related experiments

PAM Fluorometry
Phytoplankton
Variable Fluorescence



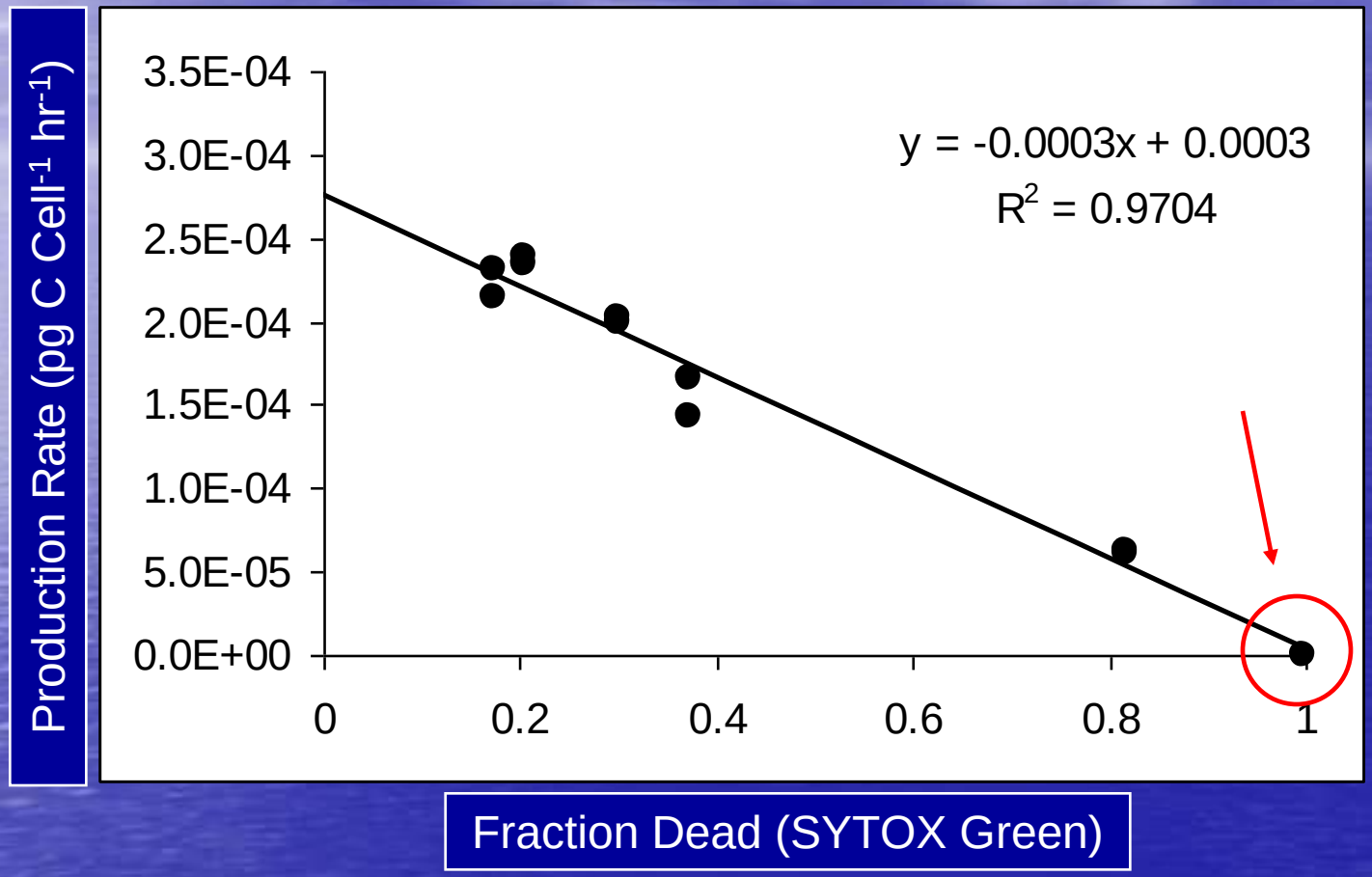


So… what do we get when we combine fancy, flow-cytometric viability analysis with bulk physiological measurements?



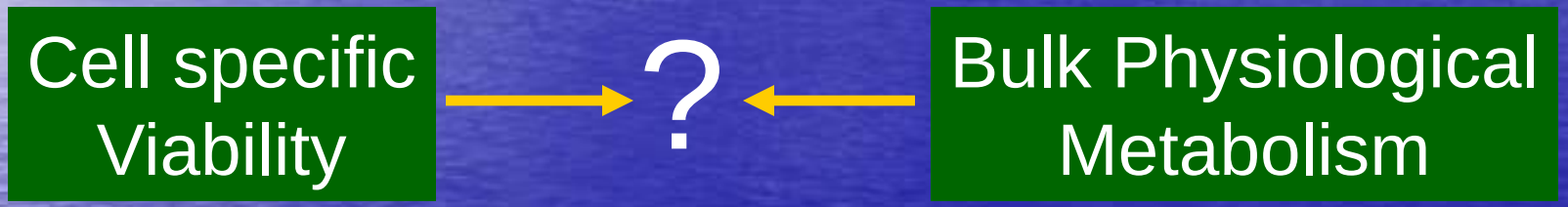
On one hand…

Metabolic verification of SYTOX Green™



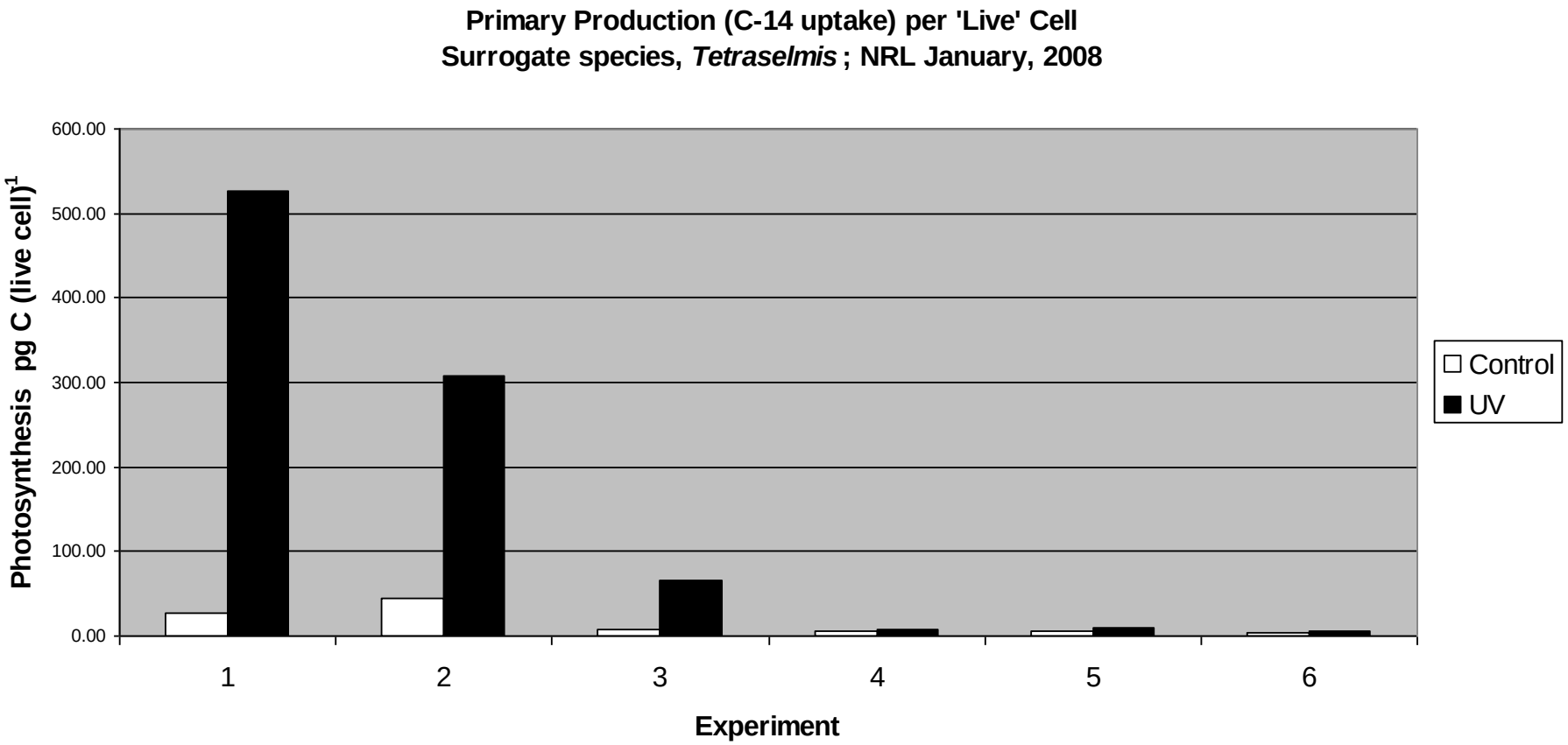
In laboratory cultures, increased membrane permeability correlates with a reduction in photosynthesis

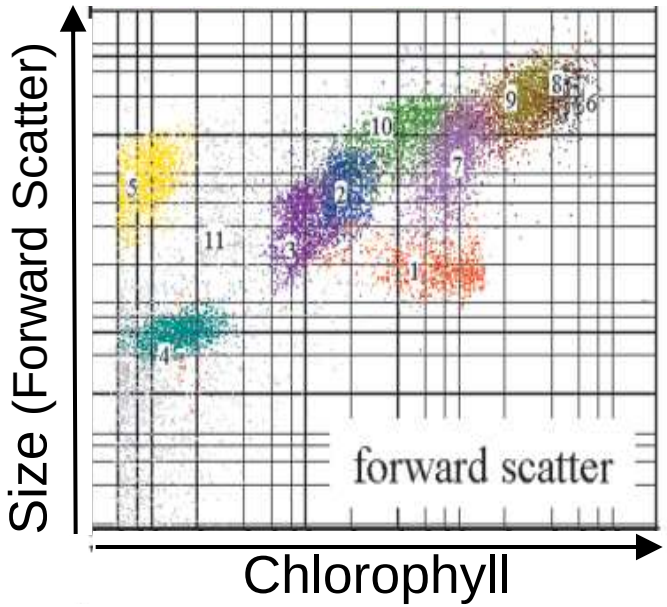
So… what do we get when we combine fancy, flow-cytometric viability analysis with bulk physiological measurements?

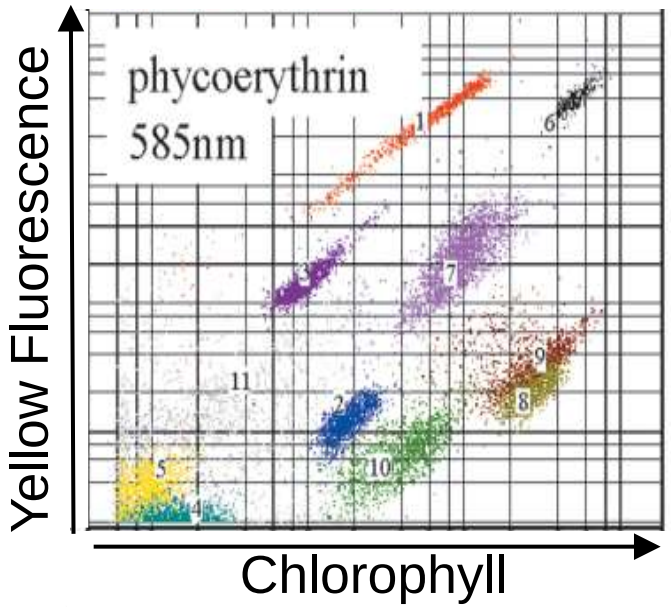


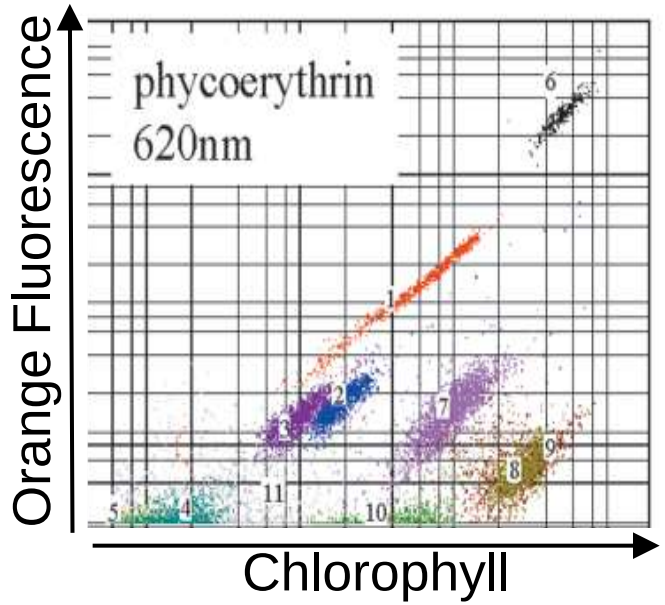
On the other hand…

Evidence of photosynthetic metabolism in ‘dead’ cells









Cyano.:	Cryp.:	PB-less eukaryotes:
1 Osci. ssp.	6 Crypt. ov.	8 Amph. kleb. (Dinoph.)
2 Mic. aer.	7 Rhod. spec.	9 Cycl. mene. (Bacill.)
3 Syn.cy. (11d)	(freshwater ref.)	10 Chlor. fusc. (Chloro.)
4 Syn.cy. (42d)		
5 Anac. nid.		detritus: 11

(Becker et al. 2002)

Conclusions:

•Determination of live and dead cell concentrations in marine microbes is an important, but evolving procedure in marine science – it is not yet perfect

•At the moment, the best and most conservative means of establishing 'ground truth' in viability studies includes bulk physiological measurements of metabolism and growth

•The combination of cell-specific viability staining with complementary physiological measurements will establish confidence in the selection of methods used to test ballast treatment efficacy

Remember: Ballast tank organisms are 'Wanted Dead – NOT Alive'