Ballast Tank Organisms: Wanted Dead – NOT Alive

Nick Welschmeyer and Sarah Smith

Moss Landing Marine Laboratories
Moss Landing CA 95039
Welschmeyer@mlml.calstate.edu

California Prevention First, Sept 10th, 2008

Support from:
• 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.

<table>
<thead>
<tr>
<th>Mesoplankton</th>
<th>Nanoplankton</th>
<th>Bacteria, Viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>International Convention for the Control and Management of Ships Ballast Water &amp; Sediments; IMO 2004</strong></td>
<td>&lt; than 10 viable organisms per m³ for those organisms &gt; 50 µm minimum dimension</td>
<td>&lt; 10 viable organisms per mL for those organisms &gt; 10 µm but &lt; 50 µm minimum dimension</td>
</tr>
<tr>
<td><strong>Ballast Water Management Act of 2005; US Senate Bill 363, February 10th, 2005</strong></td>
<td>&lt; than 0.1 viable organisms per m³ for those organisms &gt; 50 µm minimum dimension</td>
<td>&lt; 0.1 viable organisms per mL for those organisms &gt; 10 µm but &lt; 50 µm minimum dimension</td>
</tr>
<tr>
<td><strong>Report and Recommendation of the California Advisory Panel on Ballast Water Performance Standards (Staff recommendations, November 2005)</strong></td>
<td>No detectable viable organisms &gt; 50 µm minimum dimension</td>
<td>&lt; 10 viable organisms per L for those organisms &gt; 10 µm but &lt; 50 µm minimum dimension</td>
</tr>
</tbody>
</table>
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?
Definition of unicellular viability

- Cells capable of growth (cell division) and metabolism

<table>
<thead>
<tr>
<th></th>
<th>Capable of growth</th>
<th>Metabolically active</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Viable but inactive</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>Dormant</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>Active – non cultivable</td>
<td>?</td>
<td>X</td>
</tr>
<tr>
<td>Dead</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

To score a cell as truly live or dead, we must measure growth capacity and metabolic activity.
Colony forming units vs. direct count

0.1-10% of direct count

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

(Daley & Hobbie 1975)
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.
<table>
<thead>
<tr>
<th>Stain/Dye</th>
<th>Assay target</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTC</td>
<td>cell respiration</td>
<td>Live cells fluoresce red</td>
</tr>
<tr>
<td>Calcein AM</td>
<td>intracellular esterase activity</td>
<td>Live cells fluoresce green</td>
</tr>
<tr>
<td>BCECF AM</td>
<td>intracellular esterase activity</td>
<td>Live cells fluoresce green</td>
</tr>
<tr>
<td>FDA</td>
<td>intracellular esterase activity</td>
<td>Live cells fluoresce green</td>
</tr>
<tr>
<td>CFDA-AM</td>
<td>intracellular esterase activity</td>
<td>Live cells fluoresce green</td>
</tr>
<tr>
<td>CDFA</td>
<td>intracellular esterase activity</td>
<td>Live cells fluoresce green</td>
</tr>
<tr>
<td>trypan blue</td>
<td>Membrane Integrity</td>
<td>Dead cells stain blue</td>
</tr>
<tr>
<td>Evan's Blue</td>
<td>Membrane Integrity</td>
<td>Dead cells stain blue</td>
</tr>
<tr>
<td>SYTOX® Green</td>
<td>Membrane Integrity</td>
<td>DNA of dead cells fluoresce green</td>
</tr>
<tr>
<td>7-AAD</td>
<td>Membrane integrity</td>
<td>DNA of dead cells fluoresce red</td>
</tr>
<tr>
<td>EthD-1</td>
<td>Membrane Integrity</td>
<td>DNA of dead cells fluoresce red-orange</td>
</tr>
<tr>
<td>PI</td>
<td>Membrane Integrity</td>
<td>DNA of dead cells fluoresce red-orange</td>
</tr>
<tr>
<td>DiOC2</td>
<td>mitochondrial membrane potential</td>
<td>Mitochondria of live cells fluoresce red</td>
</tr>
<tr>
<td>TTC</td>
<td>redox potential</td>
<td>medium fluoresces red with live cells</td>
</tr>
<tr>
<td>XTT</td>
<td>redox potential</td>
<td>medium turns orange</td>
</tr>
<tr>
<td>MTT</td>
<td>redox potential</td>
<td>medium turns purple</td>
</tr>
<tr>
<td>resazurin</td>
<td>Unid. intracellular enzymatic/chemical activity</td>
<td>medium fluoresces pink in presence of live cells</td>
</tr>
<tr>
<td>alamarBlue™</td>
<td>Unid. intracellular enzymatic/chemical activity</td>
<td>medium fluoresces pink in presence of live cells</td>
</tr>
</tbody>
</table>
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.

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

<table>
<thead>
<tr>
<th>Detector</th>
<th>Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Scatter</td>
<td>Size</td>
</tr>
<tr>
<td>Side Scatter</td>
<td>Shape</td>
</tr>
<tr>
<td>Red Fluorescence</td>
<td>Chlorophyll autofluorescence</td>
</tr>
<tr>
<td>Orange Fluorescence</td>
<td>Phycobilin autofluorescence</td>
</tr>
<tr>
<td>Green Fluorescence</td>
<td>Stains mild autofluorescence</td>
</tr>
</tbody>
</table>

A sample of fluid is introduced into the cytometer along with a separate sheath fluid, which helps in hydrodynamic focusing. The sample gets sheared by this fluid and enters the cytometer where it is focused using a laser. The sample is then sorted through a series of detectors:

- **Forward Scatter** measures the size of the sample.
- **Side Scatter** measures the shape of the sample.
- **Red Fluorescence** detects Chlorophyll autofluorescence.
- **Orange Fluorescence** detects Phycobilin autofluorescence.
- **Green Fluorescence** detects Stains with mild autofluorescence.

The cytometer also sorts the sample into a waste stream.
Flow Cytometric Analysis of Viability – Sytox Green

Live Phaeodactylum

Dead Phaeodactylum (Glutaraldehyde)

Live - Dead

Red

Green

Red

Green
FDA staining of natural phytoplankton (Elkhorn Slough)

Cryptophytes

Forward Scatter

Chlorophyll

Green Fluorescence

Counts

Forward Scatter

Chlorophyll

Green Fluorescence

Counts

FDA++

FDA+

FDA−

Unstained
FDA Staining Results: Continued

Station 10
Station 9
Station 8
Station 7
Station 6
Station 5
Station 2
Station 1

1600 cells ml⁻¹

% in metabolic category

Station

Cells per ml (×1000)

Station

Percentage in metabolic category

Stations 1 to 10:
- FDA-
- FDA+
- FDA++
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

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

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

Microbial (<50um) ATP content

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

Rau et al (in prep). CO$_2$ as a biocide; 7 days after 25% CO treatment ended.

Photosynthetic C-14 uptake

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

On one hand…
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…

Cell specific Viability  ❓  Bulk Physiological Metabolism
Evidence of photosynthetic metabolism in ‘dead’ cells

Primary Production (C-14 uptake) per 'Live' Cell

![Graph showing primary production (C-14 uptake) per live cell.](image-url)
(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’