Notes for this slide

A Revised Assessment of the Most Probable Number (MPN) Method for Enumerating Viable Phytoplankton Cells in Ballast Water Discharge

John J. Cullen and Hugh L. MacIntyre

Department of Oceanography, Dalhousie University, Halifax, NS, Canada

19th International Conference on Aquatic Invasive Species
April 10-14, 2016
Winnipeg, MB Canada

Supported by:

DALHOUSIE UNIVERSITY *Inspiring Minds*

NSERC CRSNG

TROJAN UV WATER CONFIDENCE™

The purpose of ballast water treatment:

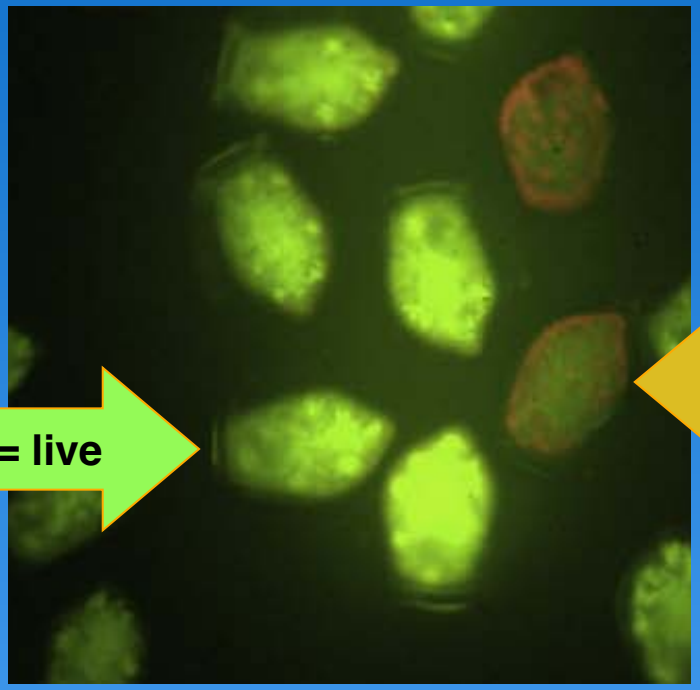
"kill, render harmless, or remove organisms"

Images: NASA, Phys.org, RiverheadLocal

Quote from US Coast Guard Final Rule, 2012

Notes for this slide

The U.S. Coast Guard Prescribes a Live / Dead Test Using Vital Stains + Motility



Stained or moving = live

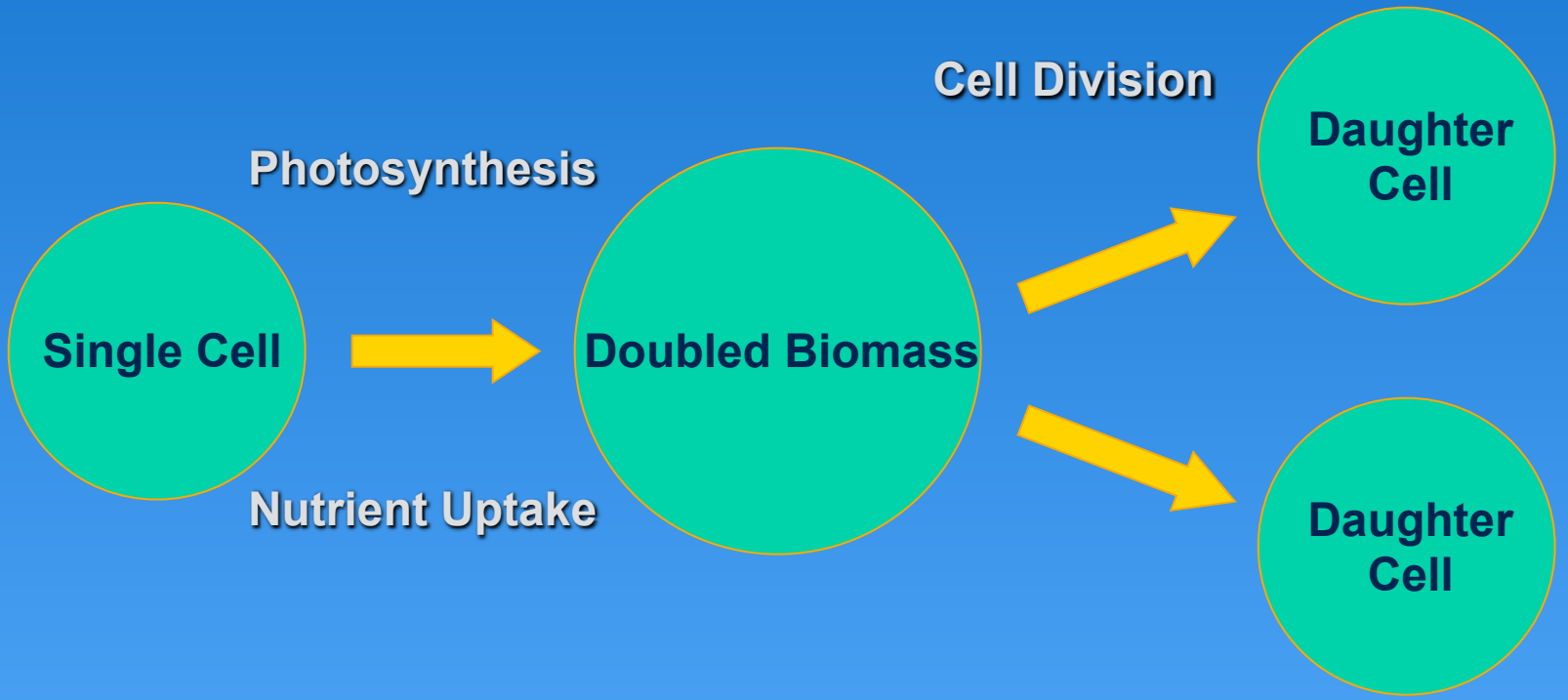
Unstained and unmoving = dead

"ETV protocol"

U.S. Environmental Protection Agency
Environmental Technology Verification Program

Notes for this slide

The Most Probable Number method enumerates viable cells, i.e., those capable of reproduction and thus invasion



Notes for this slide

MPN (viable cells) was rejected as an equivalent alternative to stains + motility (living cells)

12/14/2015: Coast Guard decision on use of Most Probable Number method

Posted by Lt. Jodie Knox, Monday, December 14, 2015

Today, the Coast Guard's Marine Safety Center informed four ultraviolet ballast water management system, or BWMS, manufacturers that the Most Probable Number, or MPN, method is not considered as an equivalent alternative to the testing method prescribed in the Coast Guard's regulations pertaining to the type approval of ballast water systems.

http://mariners.coastguard.dodlive.mil/2015/12/14/12142015-coast-guard-decision-on-use-of-most-probable-number-method/

Key argument 1:

Notes for this slide

Modern techniques are considered reliable and accurate

"U.S. type-approval procedures specify a method for determining the number of "living" organisms. Modern techniques currently exist to make this live/dead judgment reliably and with a high degree of accuracy."

COAST GUARD MARITIME COMMONS

THE COAST GUARD BLOG FOR MARITIME PROFESSIONALS

http://mariners.coastguard.dodlive.mil/2015/12/07/1272015-ballast-water-living-vs-viable/

Notes for this slide

The MPN method is subject to significant technical challenges

"… the most important being that it may not be possible to culture all of the types of organisms found in ballast water; simply put, we do not yet know how to consistently induce them to reproduce in the laboratory."

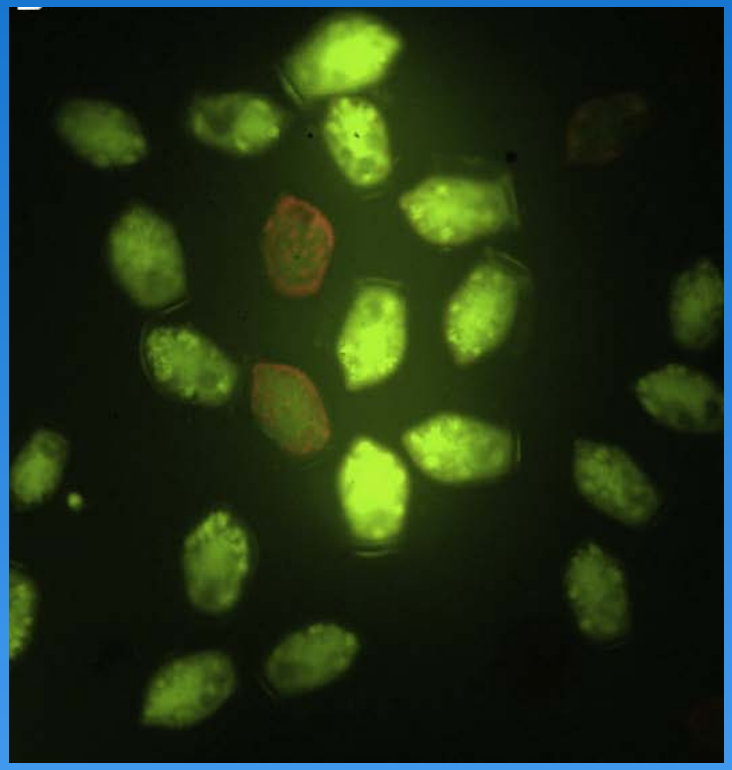
COAST GUARD MARITIME COMMONS

THE COAST GUARD BLOG FOR MARITIME PROFESSIONALS

http://mariners.coastguard.dodlive.mil/2015/12/07/1272015-ballast-water-living-vs-viable/

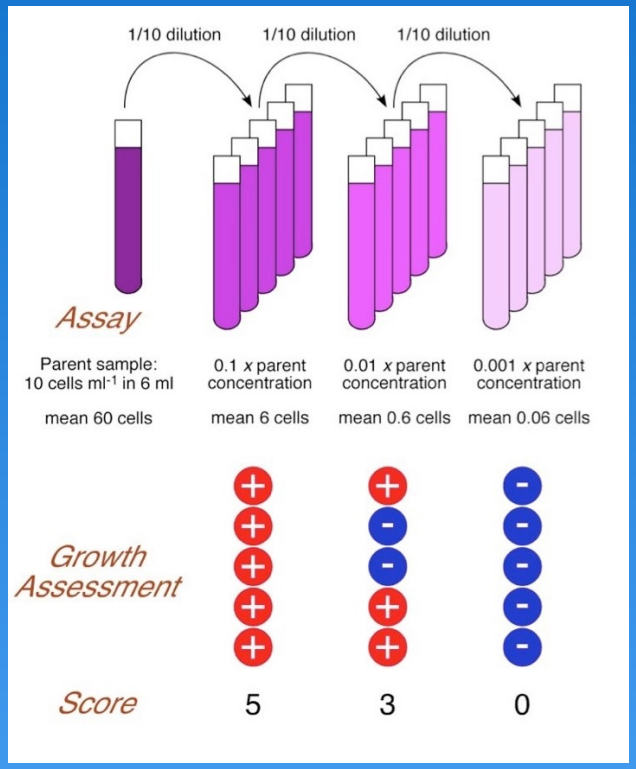
Notes for this slide

Live vs. Dead Stains + Motility



Fine

Viability from MPN

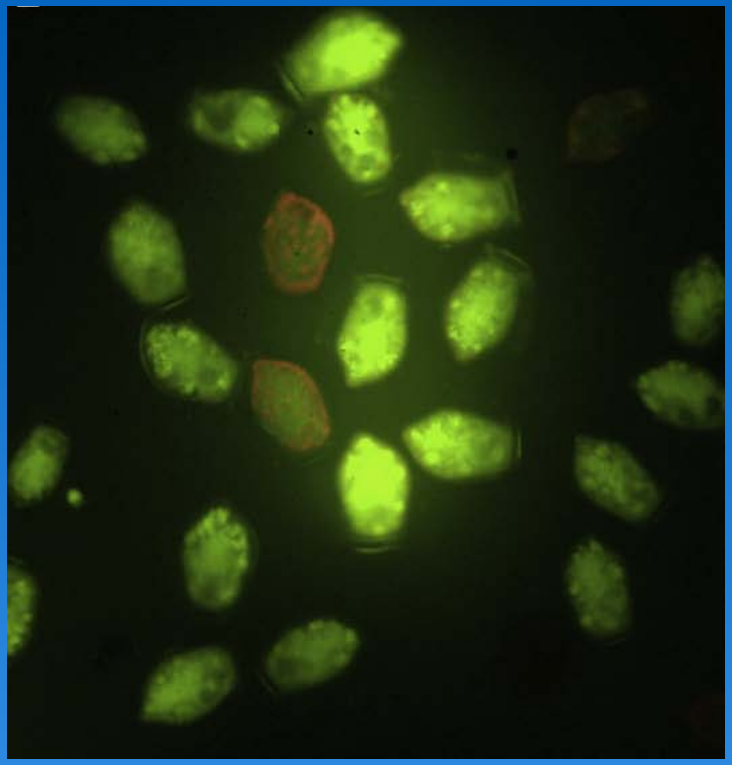


Flawed

From **Fux et al. (2010) *Toxicon* 56:1487-96.**

Notes for this slide

A fresh look at vital stains



Journal of Phycology (in press)

J. Phycol. *, ***–*** (2016)

DOI: 10.1111/jpy.12415

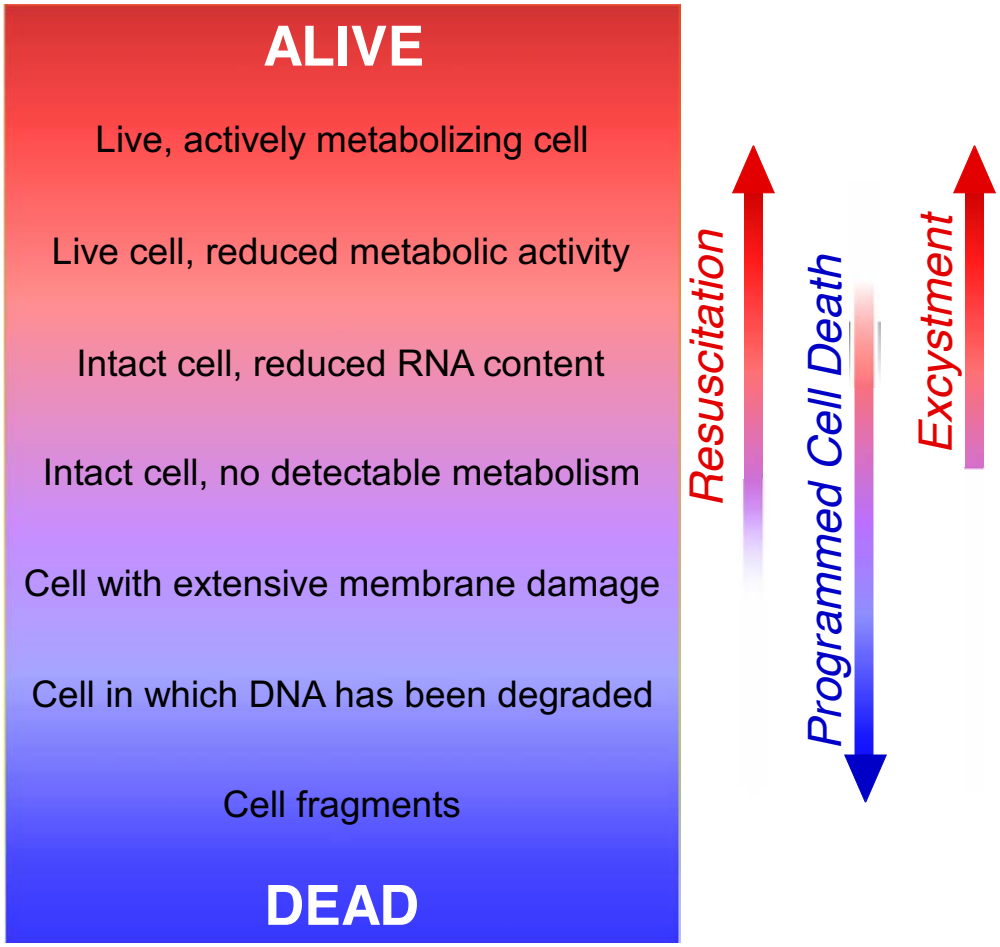
CLASSIFICATION OF PHYTOPLANKTON CELLS AS LIVE OR DEAD USING THE VITAL STAINS FLUORESCEIN DIACETATE AND 5-CHLOROMETHYLFLUORESCEIN DIACETATE[1]

Hugh L. MacIntyre[2] and John J. Cullen

Notes for this slide

There is no simple definition of live vs. dead microbes

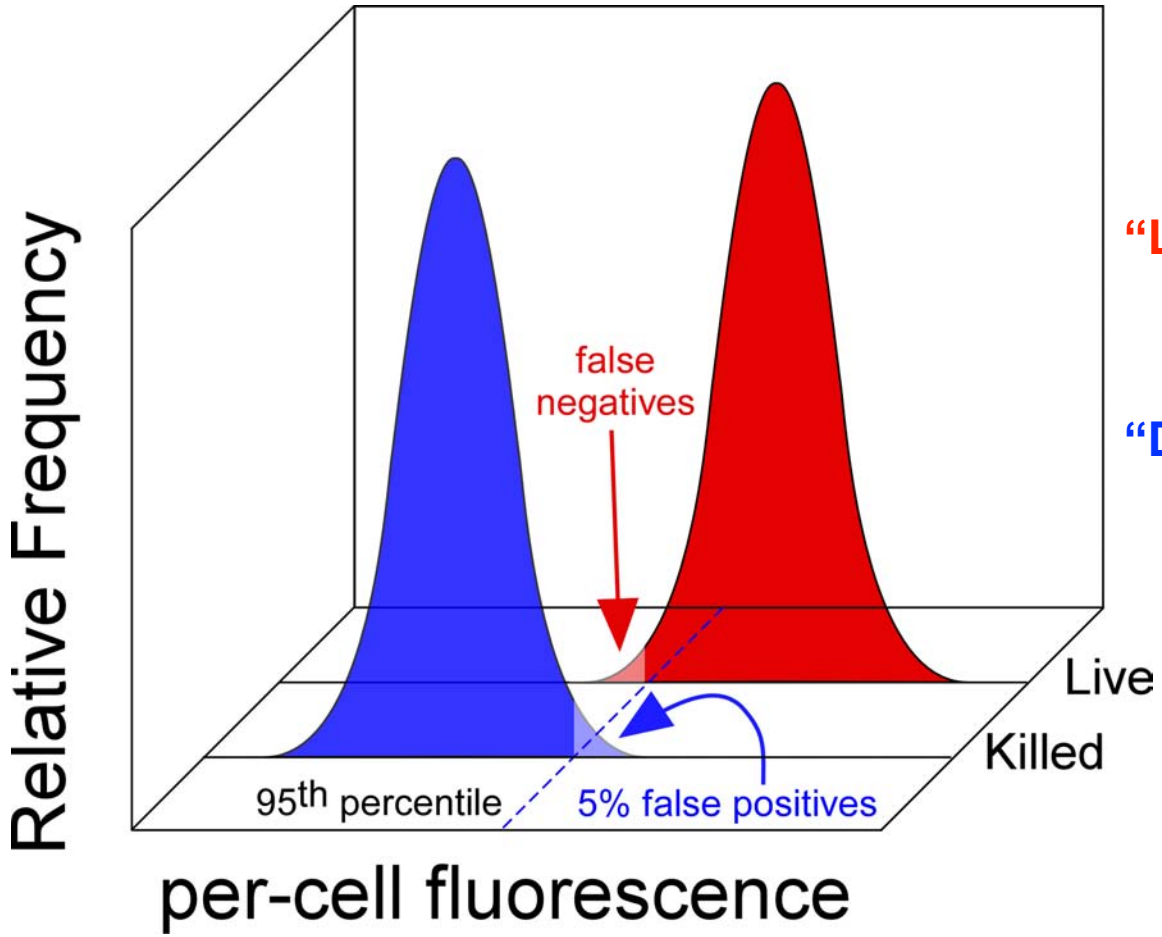
Exact point of death unknown



Based on Davey 2011

Notes for this slide

Vital stains depend on a clear separation of live vs. dead cells

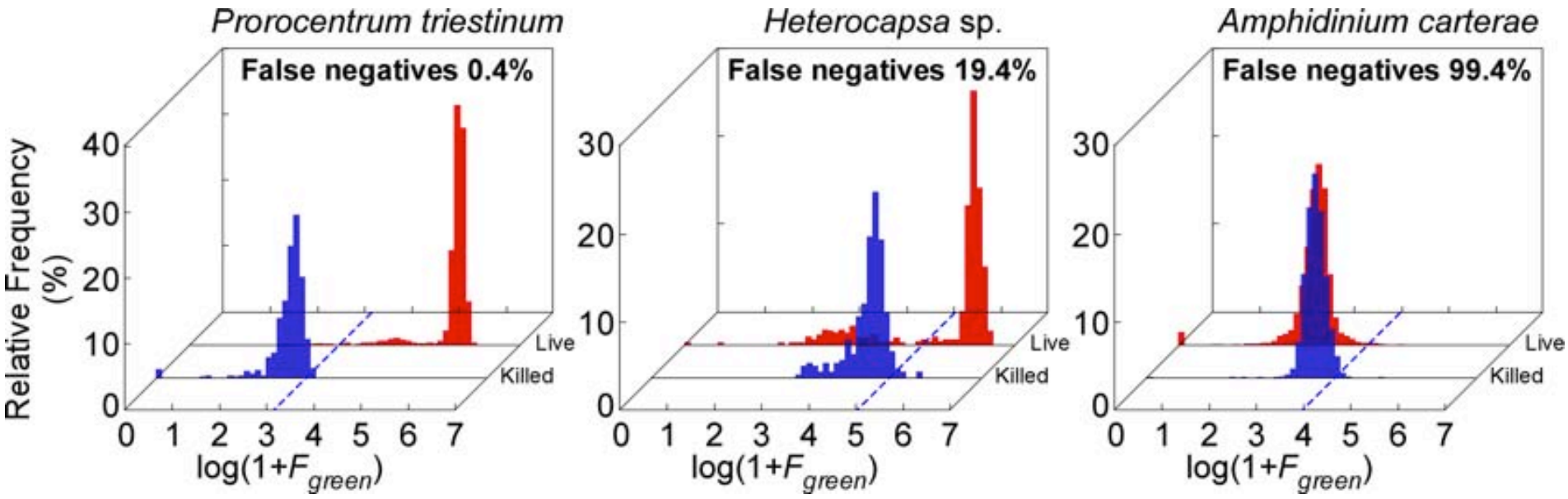


"Living" cells shown in red

"Dead" cells shown in blue

Notes for this slide

FDA + CMFDA stains work for some species but not for others



Signal is stain fluorescence measured with flow cytometry (F_{green})

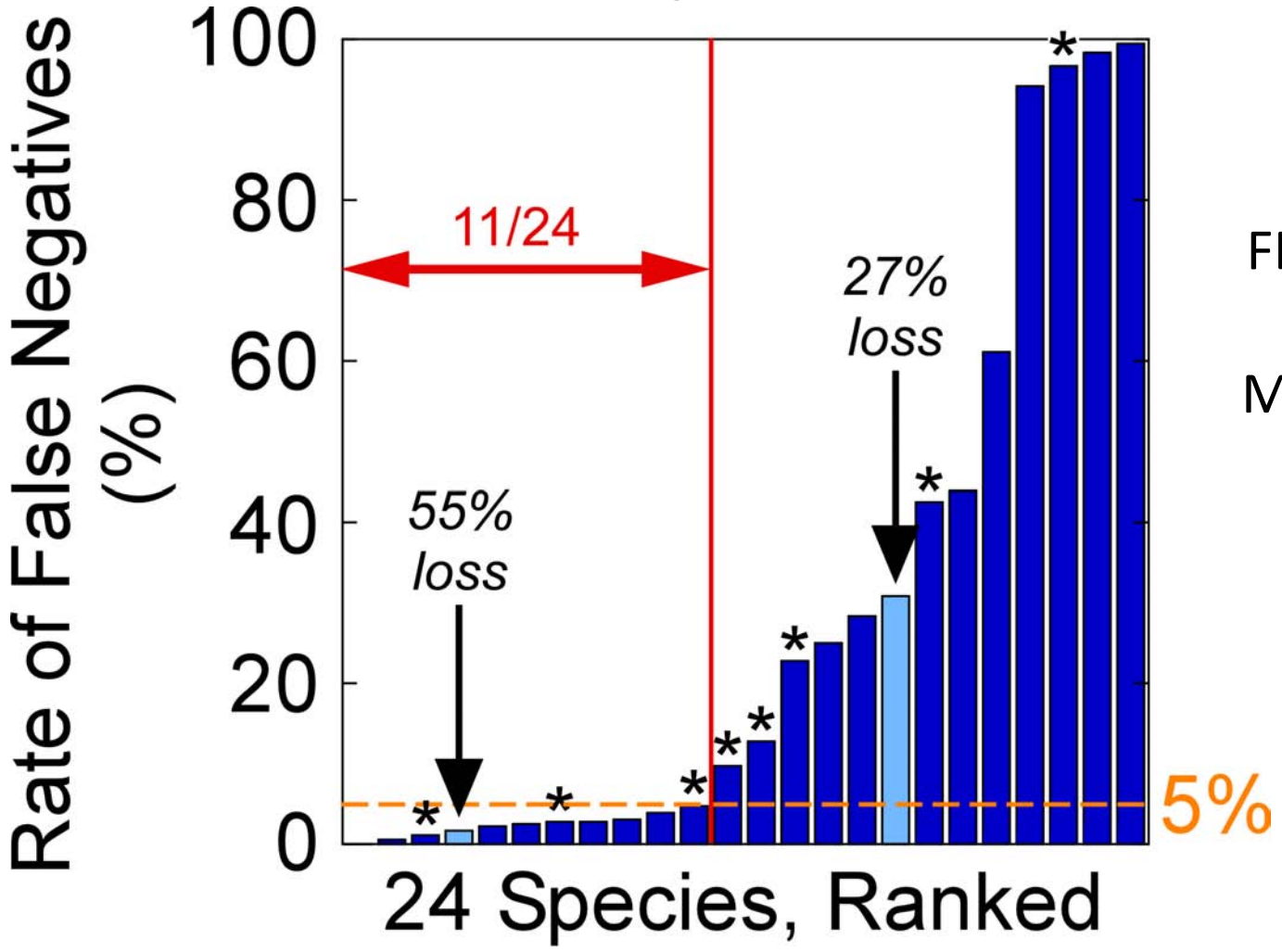
Heat-killed cells shown in blue

Exponentially-growing, viable cells in red

Notes for this slide

A minority of 24 cultured species were classified with < 10% error

* Harmful Algal Bloom species

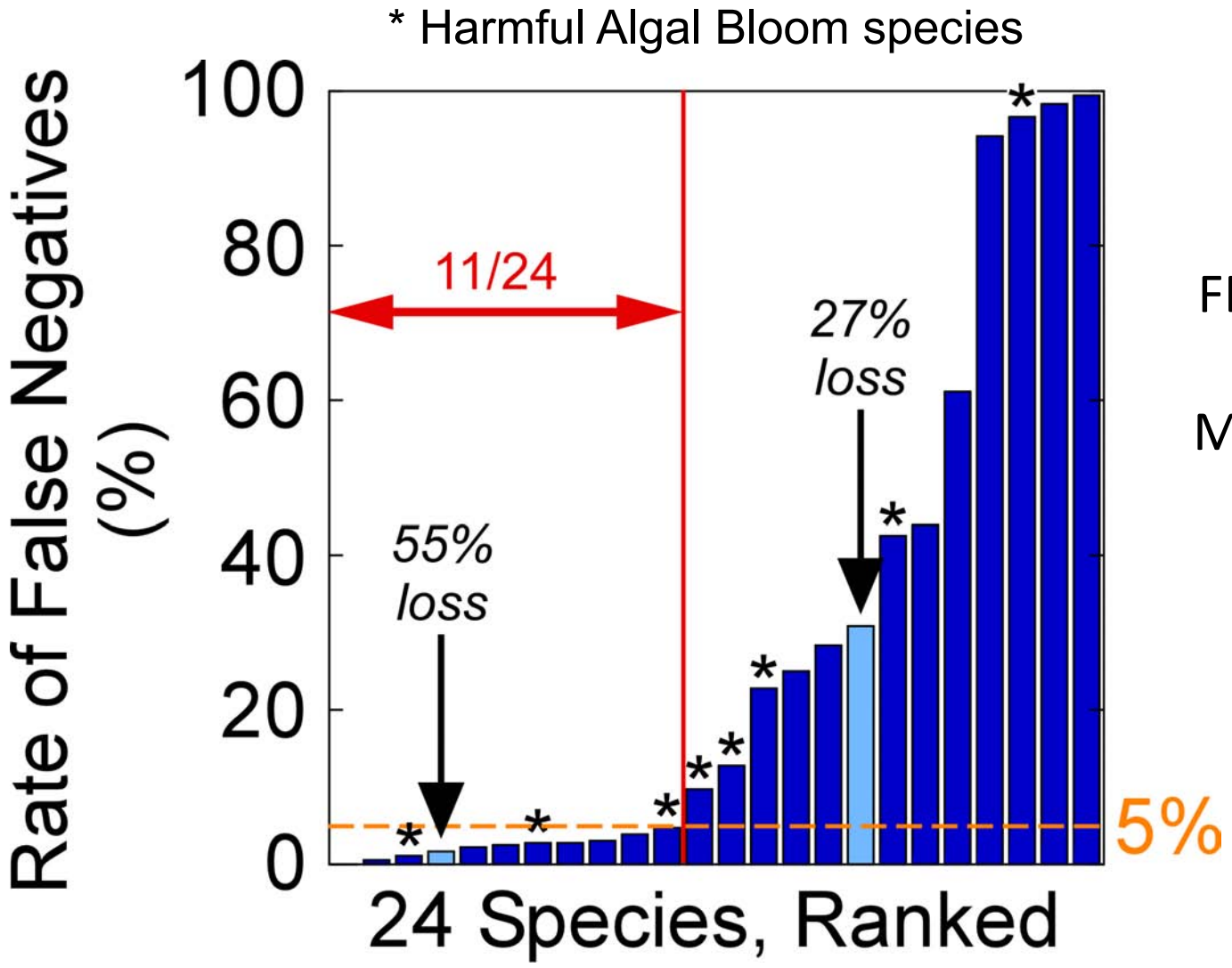


FDA+CMFDA

Means of 3-5 replicates

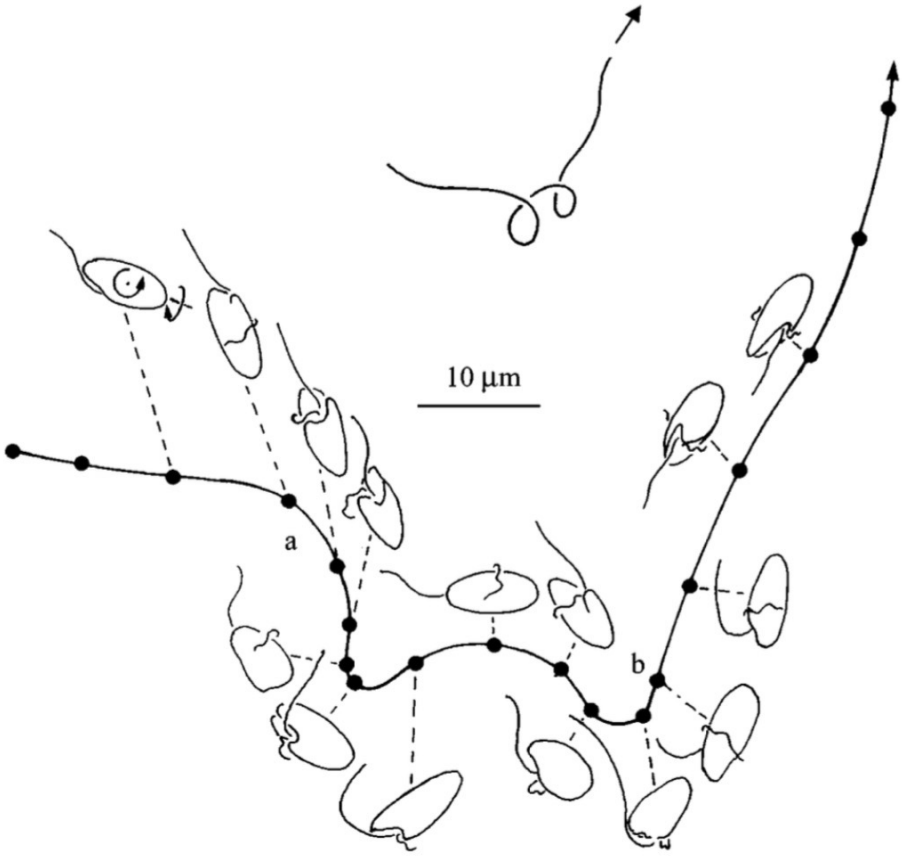
Notes for this slide

Firm conclusion: These vital stains cannot be considered accurate for all species of phytoplankton



Notes for this slide

Motile cells are also classified as living



Example of a helical swimming path in the Dinoflagellate Oxyrrhis marina (Fenchel 2001)

Notes for this slide

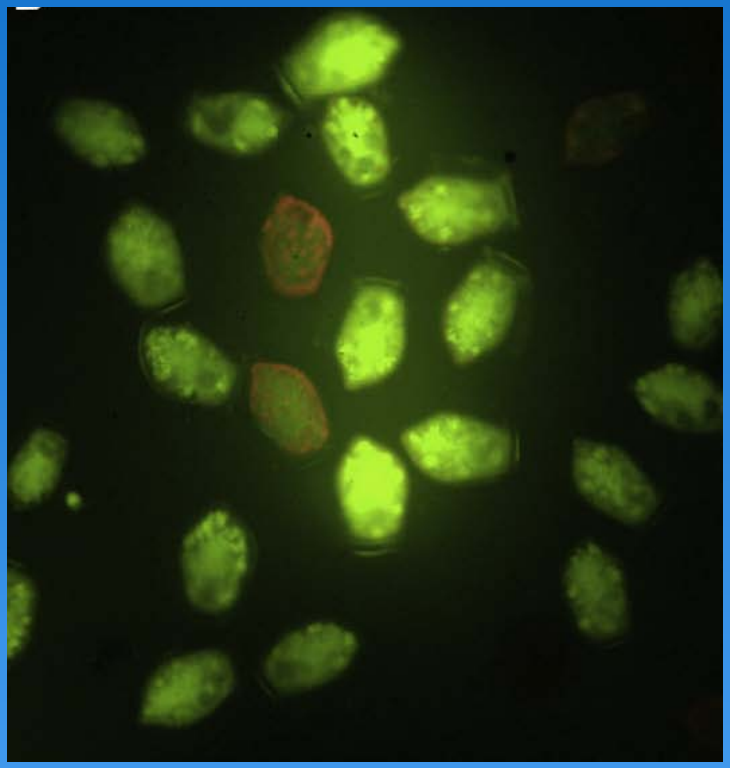
But many phytoplankton are incapable of movement

Simulated video of motility in *Thalassiosira weissflogii*

CCMP 1336 from NCMA - Bigelow Lab

Notes for this slide

Live vs. Dead Stains + Motility

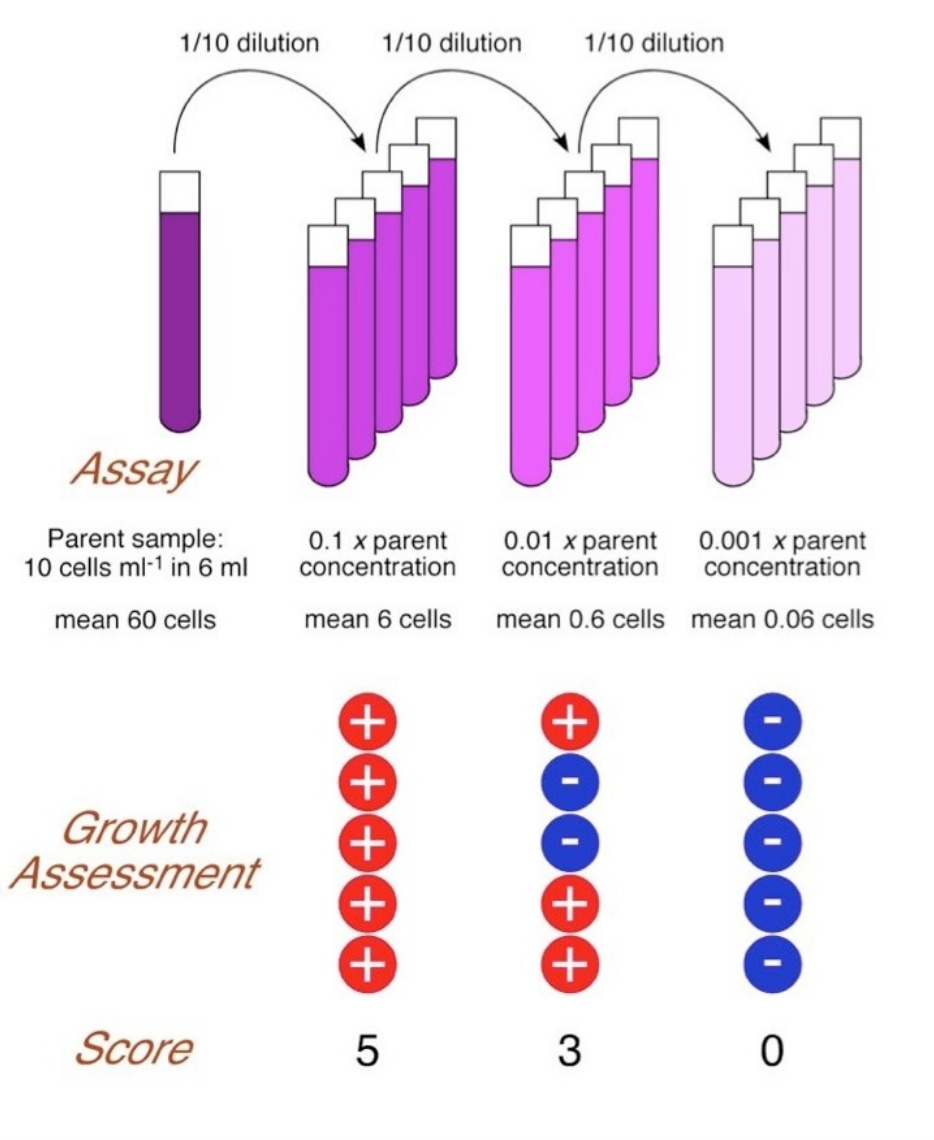


Flawed

From Fux et al. (2010) *Toxicon* 56:1487-96.

Notes for this slide

A Fresh Look at MPN



Assumptions:

- organisms are randomly distributed in each tube and evenly distributed between subsamples
- growth will be reliably detected in any tube containing one or more viable phytoplankton cells

Notes for this slide

Longstanding criticism of MPN: Many species cannot be cultured

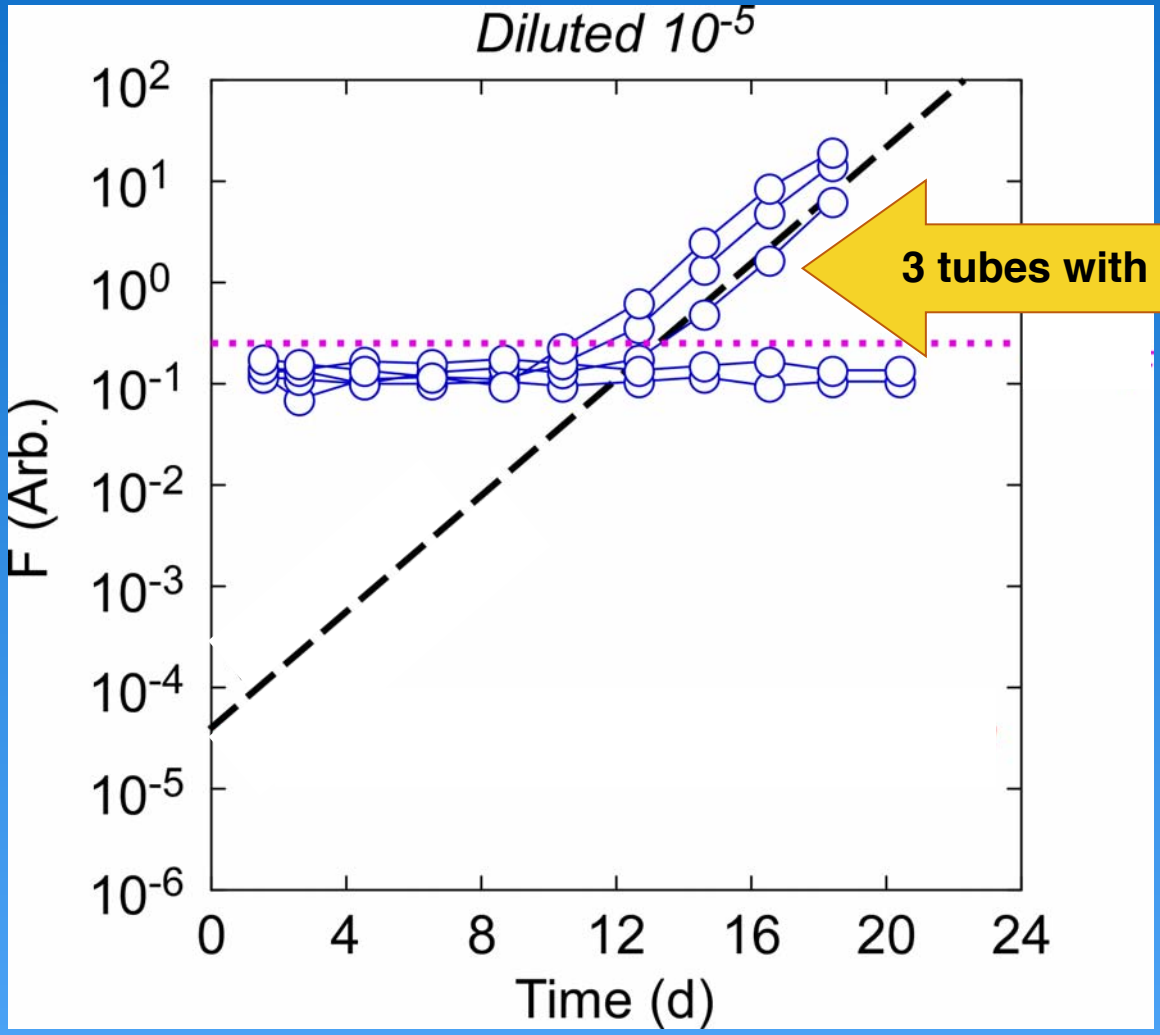
Isolating "Uncultivable" Microorganisms in Pure Culture in a Simulated Natural Environment

T. Kaeberlein, K. Lewis,* S. S. Epstein*†

The majority (>99%) of microorganisms from the environment resist cultivation in the laboratory.

Notes for this slide

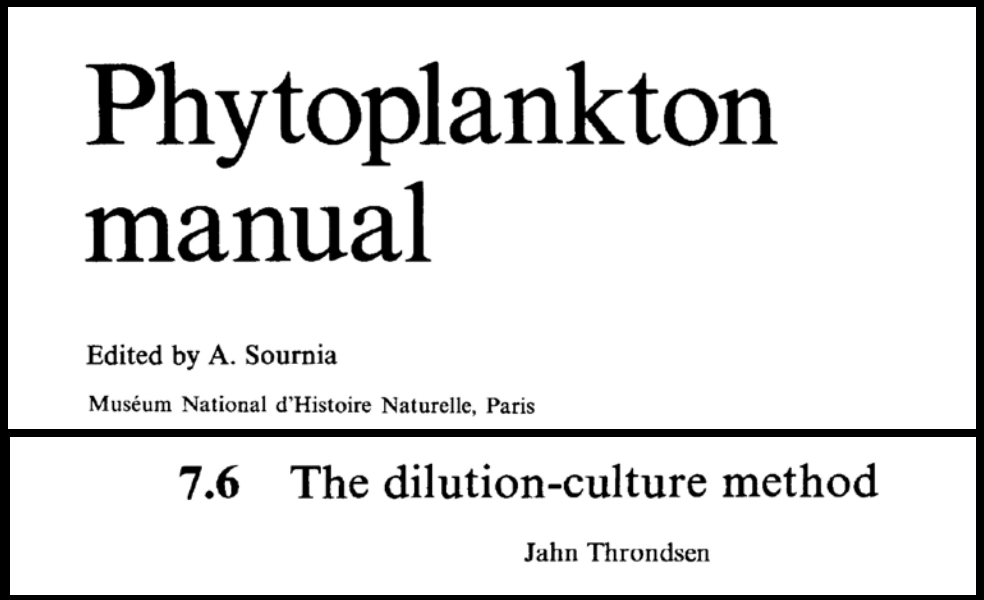
A viable cell need divide only enough times to be detected in a single growth cycle



3 tubes with one or more viable cells

Fluorescence measured in 5 tubes from a dilution series. Three tubes had one or more viable cells, two had none.

Notes for this slide



Phytoplankton manual

Edited by A. Sournia

Muséum National d'Histoire Naturelle, Paris

7.6 The dilution-culture method

Jahn Throndsen

"Some species with special requirements will regularly grow up in dilution cultures though they will not survive subculturing."

Jann Throndsen, 1978 – UNESCO Phytoplankton Manual

Notes for this slide

Many organisms can be kept for a while
Harder to maintain for years

http://blog.extension.uconn.edu/wp-content/uploads/sites/419/2013/12/house-plant-Oregon.jpg

Notes for this slide

Other issues have recently been addressed

J Appl Phycol (2016) 28:279–298
DOI 10.1007/s10811-015-0601-x

On the use of the serial dilution culture method to enumerate viable phytoplankton in natural communities of plankton subjected to ballast water treatment

John J. Cullen[1] · Hugh L. MacIntyre[1]

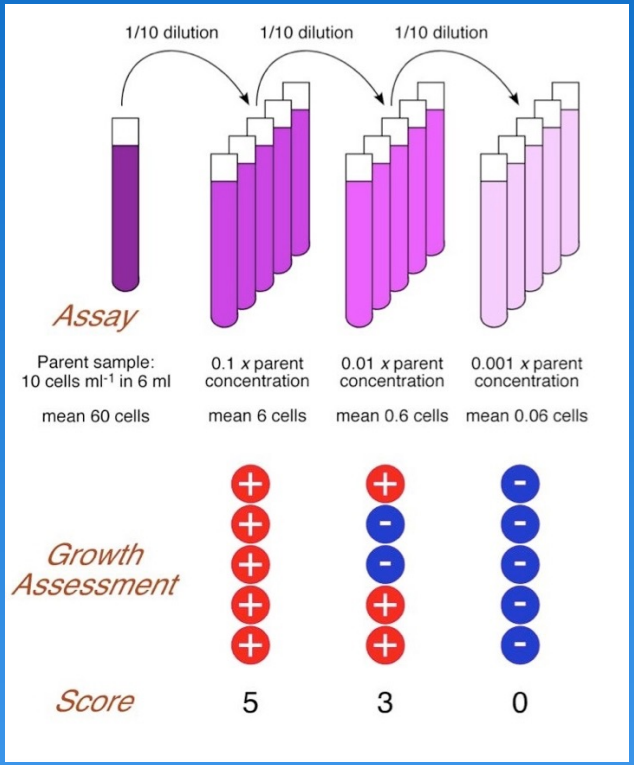
Journal of Applied Phycology – Open Access

- Grazing
- Competition
- Optimizing growth conditions
- Quantifying uncertainty

Conclusion:

Notes for this slide

Viability from MPN



With careful evaluation, potentially effective

Notes for this slide

Repair and delayed "re-growth"

Finally, it is not clear that organisms rendered nonviable will remain so over time. It has been shown that some organisms have repair mechanisms that can undo damage caused by ultra-violet radiation and thus restore the ability to reproduce.

COAST GUARD MARITIME COMMONS

THE COAST GUARD BLOG FOR MARITIME PROFESSIONALS

http://mariners.coastguard.dodlive.mil/2015/12/07/1272015-ballast-water-living-vs-viable/

Notes for this slide

Repair Processes are well known

UV-induced DNA damage and repair: a review

Rajeshwar P. Sinha and Donat-P. Häder*

Institut für Botanik und Pharmazeutische Biologie, Friedrich-Alexander-Universität, Staudtstr. 5, D-91058 Erlangen, Germany. E-mail: dphaeder@biologie.uni-erlangen.de

Received 1st February 2002, Accepted 4th February 2002
First published as an Advance Article on the web 13th March 2002

PPS Perspective
www.rsc.org/pps

Aquatic Invasions (2012) Volume 7, Issue 1: 29–36
doi: 10.3391/ai.2012.7.1.004 (Open Access)

Proceedings of the 17th International Conference on Aquatic Invasive Species (29 August–2 September 2010, San Diego, USA)

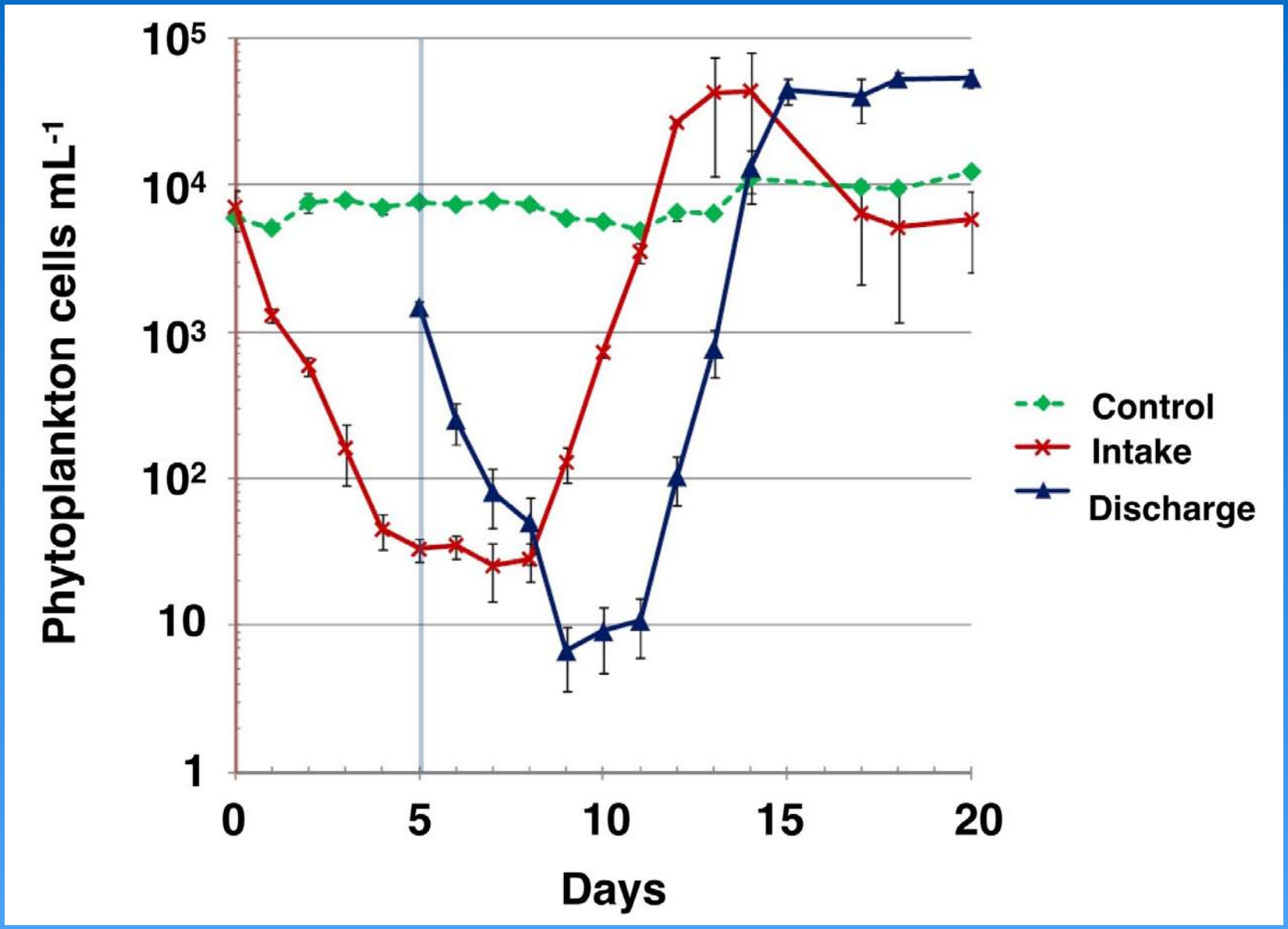
Research Article

Re-growth of potential invasive phytoplankton following UV-based ballast water treatment

Viola Liebich*, Peter Paul Stehouwer and Marcel Veldhuis

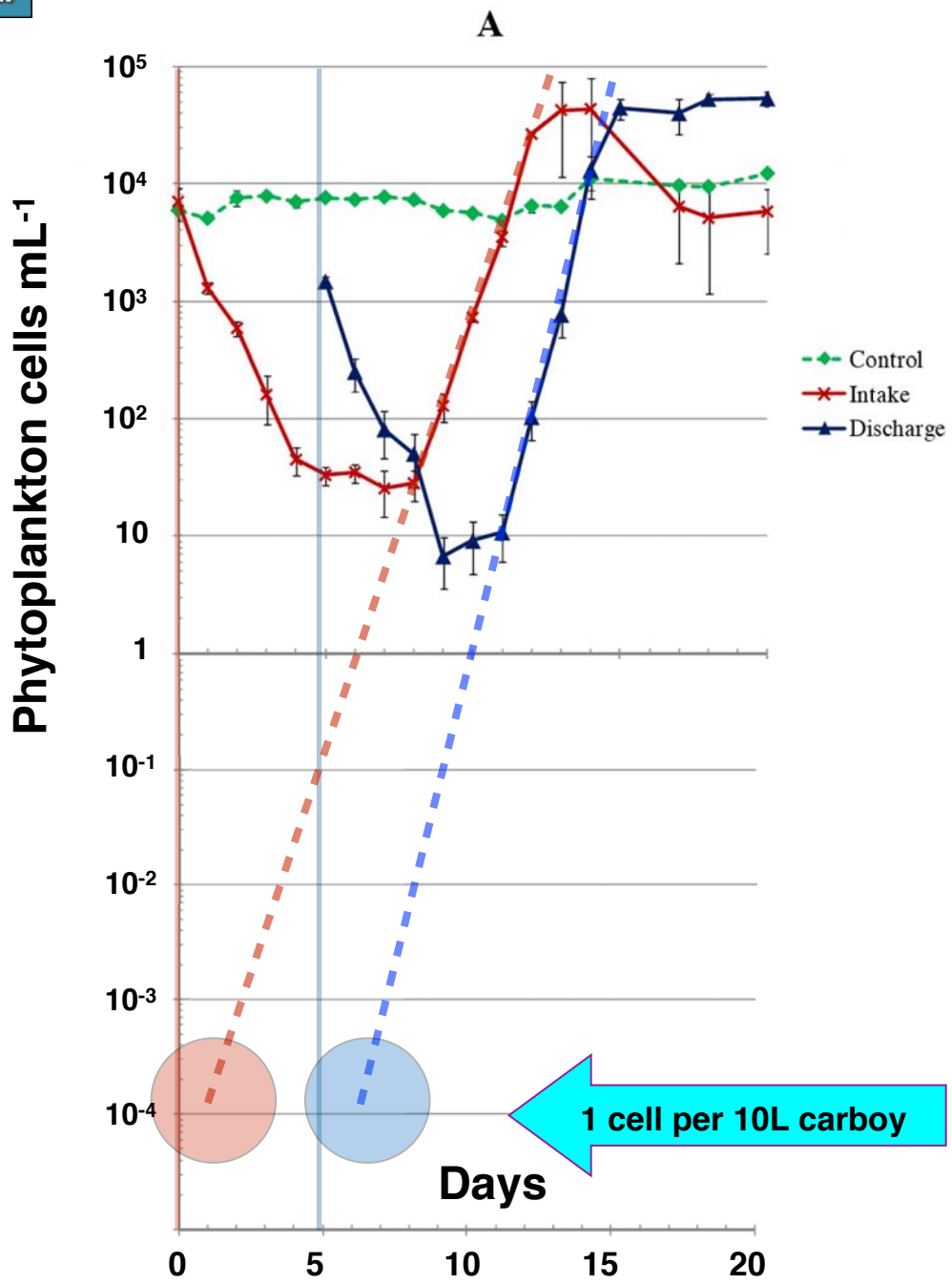
Notes for this slide

A delayed response — after a week or more?



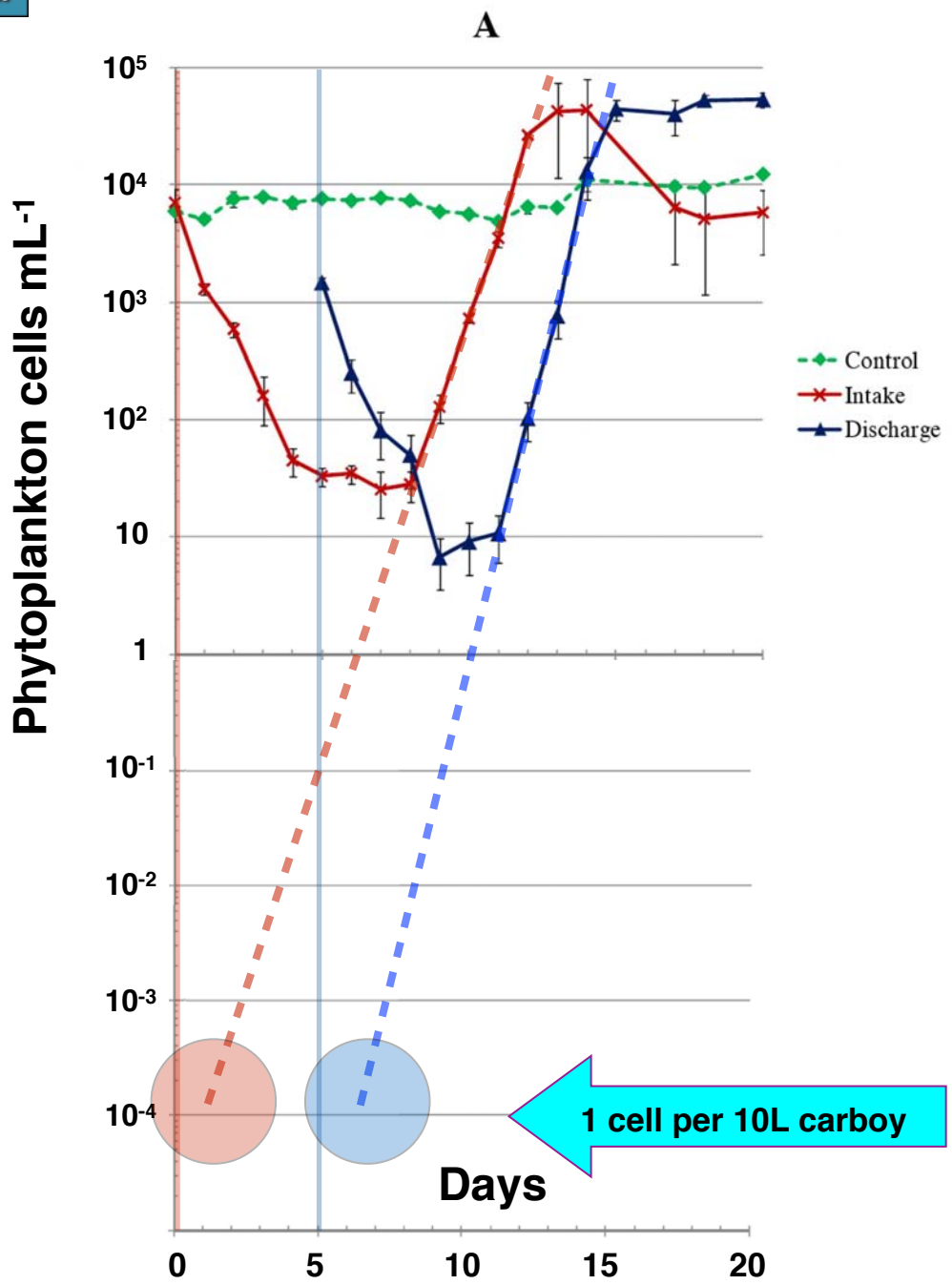
Liebich et al 2012

Notes for this slide



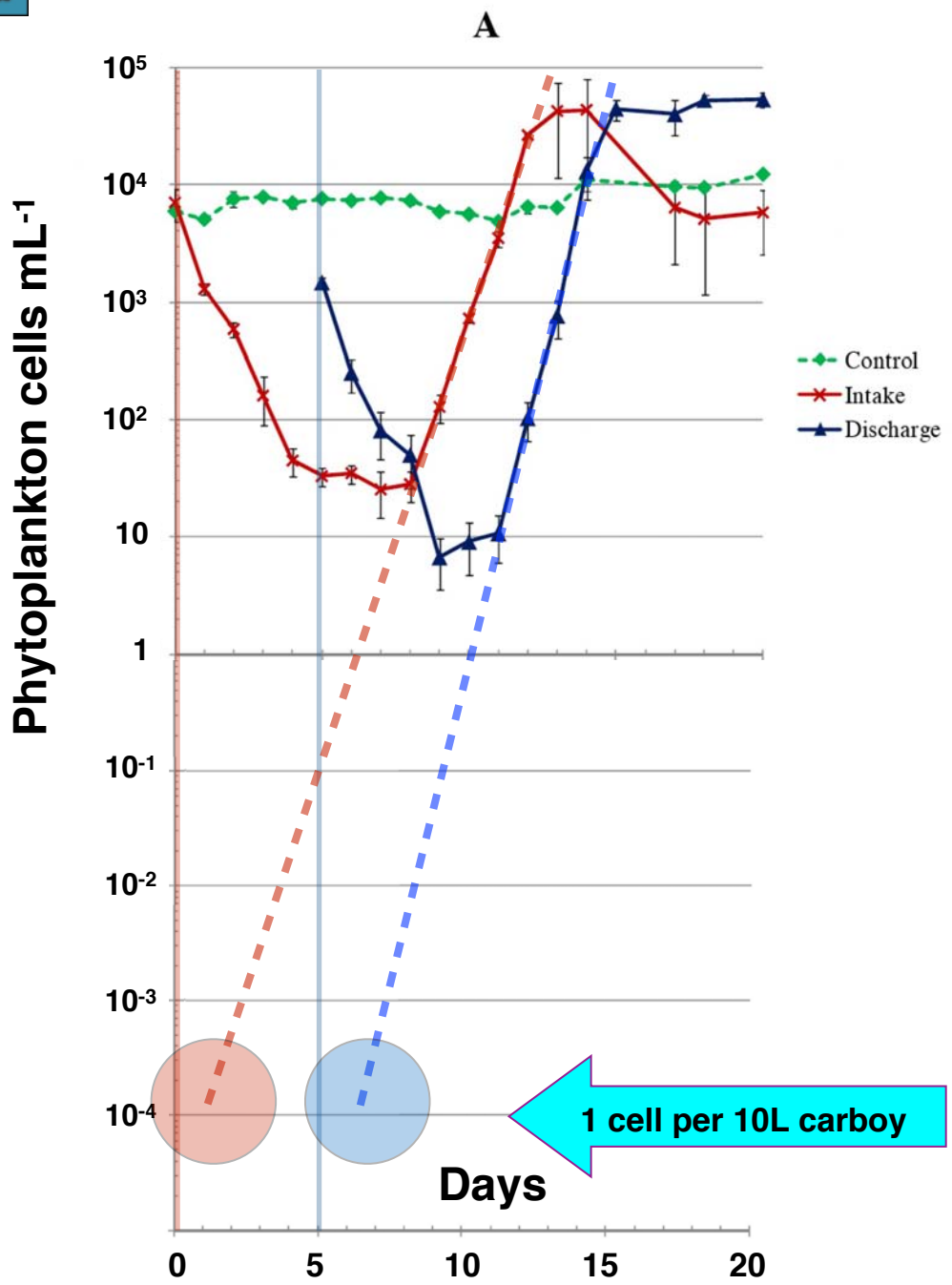
Apparent re-growth after a delay of 6-8 days is consistent with rapid repair and recovery of a small number of survivors, undetectable until numbers increase

Notes for this slide



Studies of DNA dimer repair do not suggest long (days) delays before repair commences

Notes for this slide



Studies of DNA dimer repair do not suggest long (days) delays before repair commences

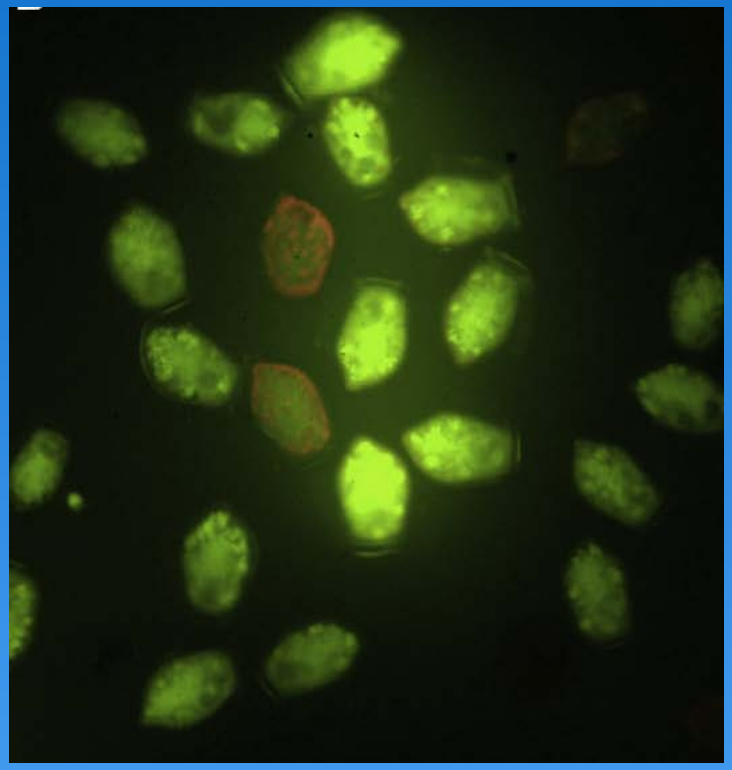
Postulate:

MPN conditions promote repair and provide enough time to detect it.

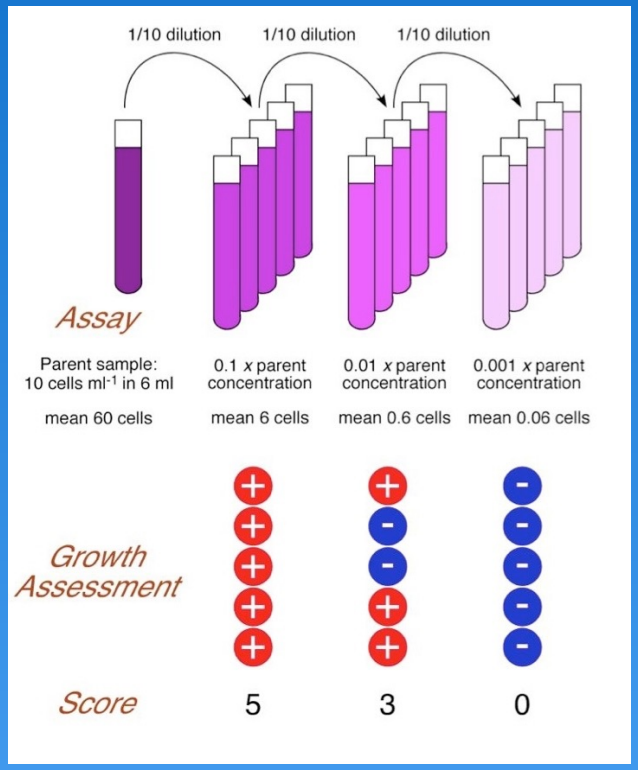
Notes for this slide

Proposed assessment based on recent research

Live vs. Dead Stains + Motility



Viability from MPN



Flawed

Comparable

From Fux et al. (2010) *Toxicon* 56:1487-96.

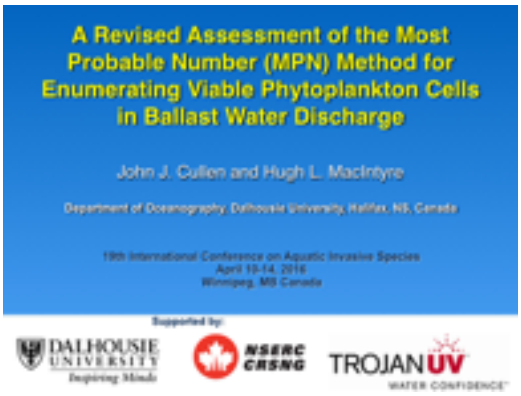
Conclusions

- The MPN method is much less vulnerable to methodological uncertainties than has been commonly thought.
- Vital stains + motility can not be considered accurate for all species of phytoplankton.

Consequently:

- With careful evaluation, MPN could serve as an effective method for assessing the viability of phytoplankton after ballast water treatment, no less protective of the environment than live/dead assessments using vital stains.

Thank you



Slide 1 Notes (*Click on image to return*)

These slides were presented on April 12, 2016 during a session on Ballast Water at the 19th International Conference on Aquatic Invasive Species in Winnipeg, Manitoba.

Peer-reviewed background materials include:

Cullen, J.J., MacIntyre, H.L., 2015. On the use of the serial dilution culture method to enumerate viable phytoplankton in natural communities of plankton subjected to ballast water treatment. Journal of Applied Phycology, 28, 279-298, DOI: 10.1007/s10811-015-0601-x

and

MacIntyre, H.L., Cullen, J.J., 2016. Classification of phytoplankton cells as live or dead using the vital stains fluorescein diacetate and 5-chloromethylfluorescein diacetate. Journal of Phycology, in press, DOI: 10.1111/jpy.12415

The links provided in the attached notes were current on April 24, 2016.

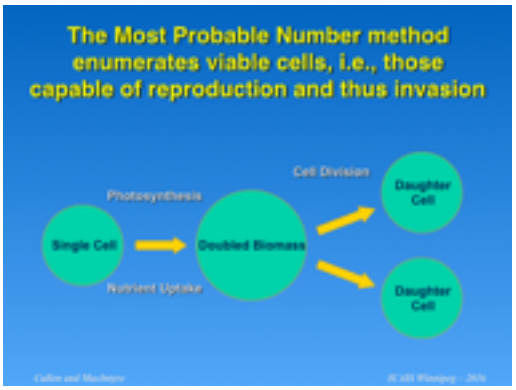
Slide 3 Notes (*Click on image to return*)

ETV Protocol

U.S. Coast Guard Final Rule

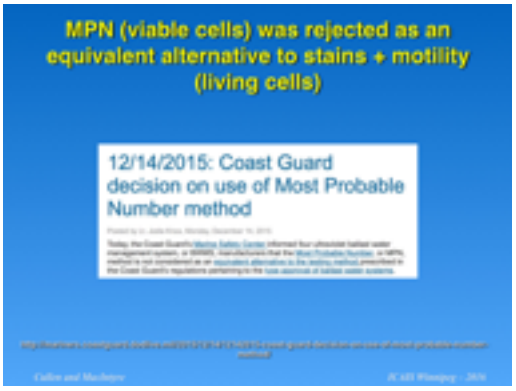
Peer-reviewed publication on the use of combined vital stains plus motility:

Steinberg, M.K., Lemieux, E.J., Drake, L.A., 2011. Determining the viability of marine protists using a combination of vital, fluorescent stains. Marine Biology, 158, 1431–1437.



Slide 4 Notes (*Click on image to return*)

The ecological importance of viability is discussed by Cullen and MacIntyre (2015), who refer to relevant scientific publications.



Slide 5 Notes (*Click on image to return*)

http://mariners.coastguard.dodlive.mil/2015/12/14/12142015-coast-guard-decision-on-use-of-most-probable-number-method/

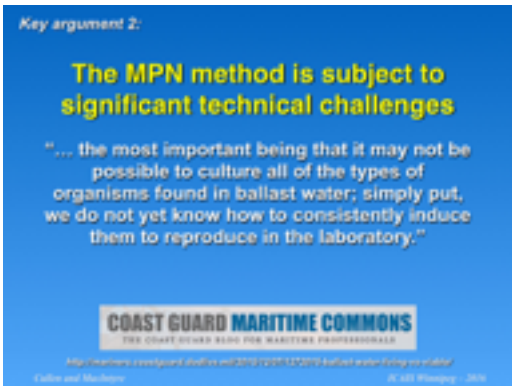
Slide 6 Notes (*Click on image to return*)

http://mariners.coastguard.dodlive.mil/2015/12/07/1272015-ballast-water-living-vs-viable/

Subsequently, on April 14, 2016, Rear Admiral Paul Thomas, testifying on behalf of the U.S. Coast Guard to the U.S. Transportation and Infrastructure Sub-Committee, made the following statement[1]:

[1:15:41] "We have an efficacy test that we know is reliable and repeatable. So the efficacy test that we have now is one that is very reliable and repeatable across a broad spectrum of ballast water that we would see from ships coming around the world, and that is the one that says we can count how many things are alive versus how many are dead."

[1] This and other quotes to follow are from Laurens, W., 2016-04-17: U.S. Debates "Ridiculous" Ballast Water Situation. http://www.maritime-executive.com/editorials/us-debates-ridiculous-ballast-water-situation. The reported times refer to a video of the testimony.

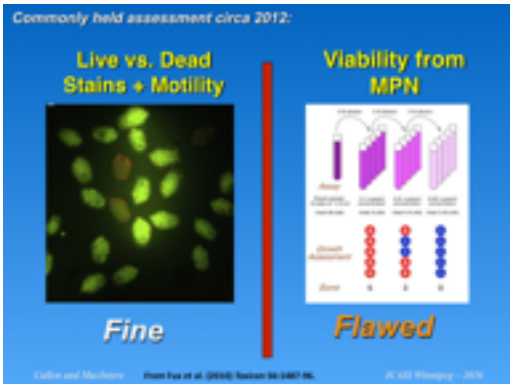


Slide 7 Notes (*Click on image to return*)

More detail is provided by Rear Admiral Paul Thomas during his April 14 testimony as reported in the article by Wendy Laurens:

[1:20:31] "The trick comes in developing the tests. The efficacy tests for the system to determine whether the system was actually killed or rendered harmless, and what I am telling you is there is a reliable repeatable efficacy test to determine if something is dead. There is not a reliable repeatable efficacy test to determine if they have been rendered harmless."

[1:21:54] "Due to the wide spectrum of species that we are talking about for ballast water all around the world, the fact that you don't even know which species you are trying to render harmless, it is difficult to prove that you've cultured enough of them to know if they are able to reproduce. That is essentially the problem."



Slide 8 Notes (*Click on image to return*)

This slide was presented to illustrate views that were commonly held in 2012 when the Coast Guard Final Rule was published. The views are consistent with recent testimony by Rear Admiral Paul Thomas

Slide 9 Notes (*Click on image to return*)

The research presented in the next few slides is described in a scientific paper that has been peer-reviewed and accepted for publication in the Phycological Society of America's *Journal of Phycology.* According to ISI Journal Citation Reports ©, the journal's ranking for 2014 is 11/103 (Marine & Freshwater Biology) and 43/204 (Plant Sciences).

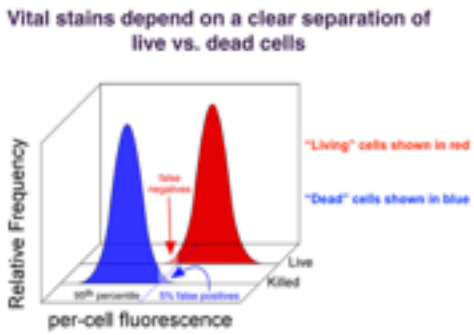
This paper is in the final stages of prepublication, and the accepted version has been posted with open access on the publisher's website (link below). Supplementary materials present results for each of the 24 species studied

MacIntyre, H.L., Cullen, J.J., 2016. Classification of phytoplankton cells as live or dead using the vital stains fluorescein diacetate and 5-chloromethylfluorescein diacetate. Journal of Phycology, in press, DOI: 10.1111/jpy.12415

Slide 10 Notes (*Click on image to return*)

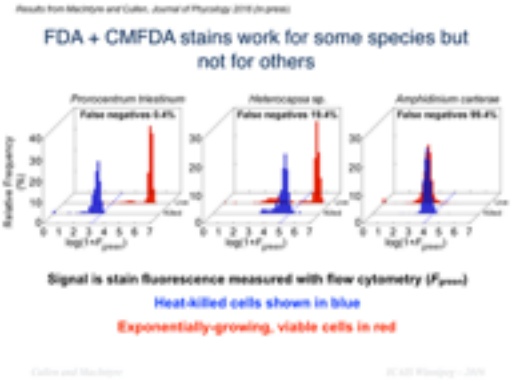
This figure is modified from Figure 1 in a relevant review:

Davey, H.M., 2011. Life, death, and in-between: meanings and methods in microbiology. Applied and Environmental Microbiology, 77, 5571-5576.



Slide 11 Notes (*Click on image to return*)

As described in MacIntyre and Cullen (2016) and many other publications, vital stains such as those prescribed by the Coast Guard are designed to reveal differences in enzymatic activity and membrane integrity, so living cells have a distinct, higher signal than dead cells. For ballast water discharge, only + or - scores are possible. A cell is classified as living, or not.

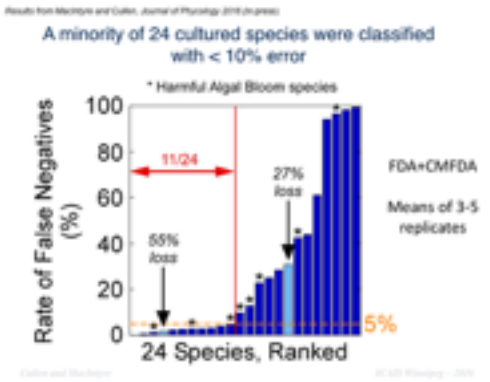


Slide 12 Notes (*Click on image to return*)

The flow cytometer measures the stain signal (green fluorescence) emitted by each cell from cultures of phytoplankton:

Live samples are shown in red. They are actively growing cultures that were demonstrated independently to be uniformly alive.

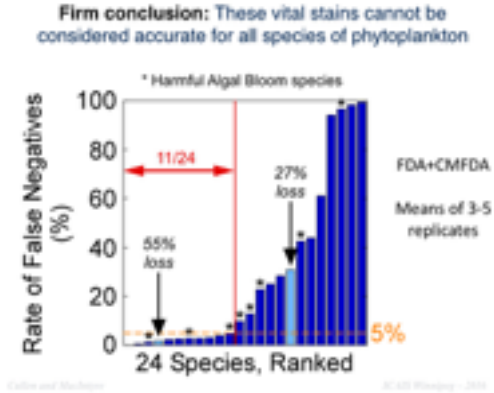
Killed samples have been heat treated and shown to be dead, as described in MacIntyre and Cullen (2016).



Slide 13 Notes (*Click on image to return*)

This graph summarizes key results of the study. Of the 24 species of phytoplankton studied, those to the left of the red line were classified as live vs. dead with < 10% error. The two pale blue bars identify species that suffered significant cell losses by the staining procedure alone, further compromising the method. Species that form harmful algal blooms are marked with an asterisk.

Note that cells were classified as dead if their fluorescence signal was less than that of 95% of the heat killed cells. High rates of false negative error — live cells classified as dead — are associated with live cells that have similar fluorescence to dead cells. If instead cells are classified as live if their signal is higher than the lowest 5% of live cells, the stains will still generate high rates of errors, but they will be false positives — dead cells classified as live.



Slide 14 Notes (*Click on image to return*)

It is rare for a scientific result to be strong enough to support an absolute statement. These results show very clearly that the stains method cannot be considered reliable and accurate for all species of phytoplankton.

The worst four species showed higher average staining in heat-killed cells than in actively growing cells. This is the opposite of expectation for the method. The abstract of MacIntyre and Cullen (2016) summarizes the findings:

ABSTRACT – Regulations for ballast water treatment specify limits on the concentrations of living cells in discharge water. The vital stains fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA) in combination have been recommended for use in verification of ballast water treatment technology. We tested the effectiveness of FDA and CMFDA, singly and in combination, in discriminating between living and heat-killed populations of 24 species of phytoplankton from 7 divisions, verifying with quantitative growth assays that uniformly live and dead populations were compared. The diagnostic signal, per-cell fluorescence intensity, was measured by flow cytometry and alternate discriminatory thresholds were defined statistically from the frequency distributions of the dead or living cells. Species were clustered by staining patterns: for 4 species, the staining of live vs. dead cells was distinct, and live-dead classification was essentially error free. But overlap between the frequency distributions of living and heat-killed cells in the other taxa led to unavoidable errors, well in excess of 20% in many. In 4 very weakly staining taxa, the mean fluorescence intensity in the heat-killed cells was higher than that of the living cells, which is inconsistent with the assumptions of the method. Applying the criteria of ≤ 5% false negative plus ≤ 5% false positive errors, and no significant loss of cells due to staining, FDA and FDA+CMFDA gave acceptably accurate results for only 8 – 10 of 24 species (i.e., 33 – 42%). CMFDA was the least effective stain and its addition to FDA did not improve the performance of FDA alone.

Slide 15 Notes (*Click on image to return*)

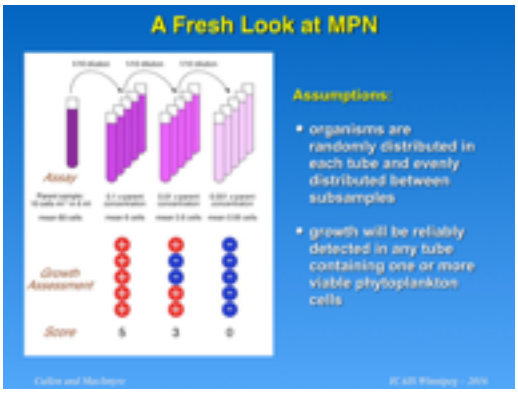
The prescribed ETV method classifies moving cells as being alive, even if they do not stain. This provides backup.

Slide 16 Notes (*Click on image to return*)

However, many species of phytoplankton are inherently incapable of movement, and others are known to stop moving when exposed to bright light under a microscope. Consequently, the stains + motility approach cannot be considered reliable for all species of phytoplankton.

Slide 17 Notes (*Click on image to return*)

Our study was accepted for publication only recently. It reinforces published accounts that called into question the accuracy of vital stains and provides a comprehensive follow-up to the FDA+CMFDA study by Steinberg et al. (2011).



Slide 18 Notes (*Click on image to return*)

The MPN method as applied to the enumeration of total viable phytoplankton is described by Cullen and MacIntyre (2015) in a review that considers much of the relevant literature, going back decades.

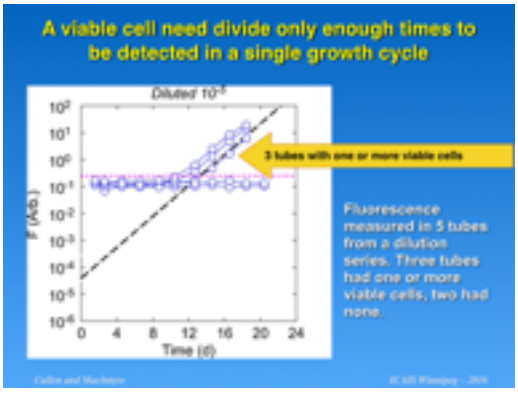
The first assumption is violated when colonial forms are encountered. The latter is commonly questioned because many species of phytoplankton have not been isolated and maintained in laboratory cultures (see slide 7).

The MPN method is also addressed in the following:

Wright, D.A., Welschmeyer, N.A., 2015. Establishing benchmarks in compliance assessment for the ballast water management convention by port state control. Journal of Marine Engineering & Technology, 14, 9-18.

Slide 19 Notes (*Click on image to return*)

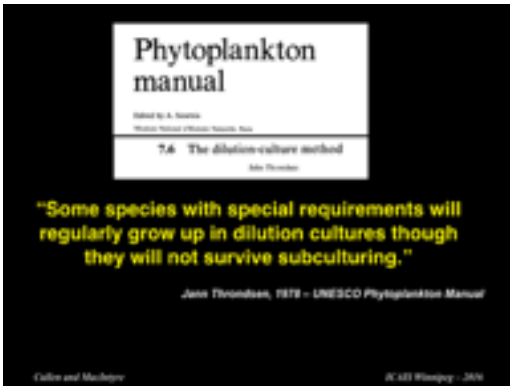
Statements such as the first sentence in this study (published in Science, 2002), have been used to support the criticism that many species of phytoplankton cannot be cultured in the MPN method. Notably, Kaeberlin, Lewis and Epstein compare their new method to growth of heterotrophic microbes in standard Petri dishes and their general characterization of the cultivation of “>99% …of microorganisms from the environment” is not supported by data in the paper.



Slide 20 Notes (*Click on image to return*)

It is true that many species of phytoplankton have not been brought into sustained culture, But importantly, the MPN method does not require “culturing” in the common sense of the word: to be assessed accurately, a single viable cell in a dilution tube need divide only enough times to be detected.

This graph from our laboratory (manuscript in review) shows results for five tubes, diluted the same and assayed for growth. The dashed line shows the estimated growth curve for a tube starting with one cell. The method depends on the viable cell growing long enough to be detected — several generations as described by Cullen and MacIntyre (2015). It is not necessary to keep the species growing indefinitely, as is required to culture phytoplankton in the commonly understood sense.



Slide 21 Notes

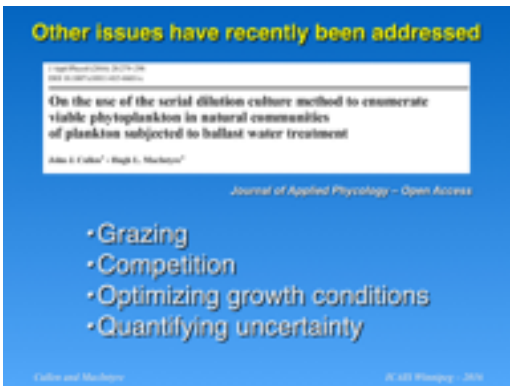
The fundamental distinction between sustained culture and growing phytoplankton in MPN was recognized long ago. As stated by the expert, Jahn Throndsen, in his review of the method:

"Some species with special requirements will regularly grow up in dilution cultures though they will not survive subculturing."

Throndsen, J., 1978. The dilution-culture method. In A. Sournia (Ed.), Phytoplankton manual, Vol. 6 (pp. 218-224). Paris: UNESCO.

Slide 22 Notes

This analogy is not perfect, but it is relevant. The requirement to grow viable phytoplankton cells to the point of detection in MPN is less of a challenge than bringing them into sustained culture.



Slide 23 Notes

Several other issues were addressed in the recent publication by Cullen and MacIntyre (2015). The discussion should be useful in further evaluations of the method.

One issue is particularly relevant to questions about how many species can be grown to detection in the MPN method:

Competition – During this talk it was noted that fast-growing and more abundant phytoplankton will dominate lists of species that are observed to grow in MPN trials. Absence from a list of "growable" species does not demonstrate that a species cannot be grown to detection in the MPN method.

Slide 24 Notes

In our peer-reviewed publication, we concluded that the MPN method is potentially effective, and we suggested several ways to quantify and minimize uncertainty. These approaches could be helpful in the Coast Guard's attempts to "determine if efficacy tests are reliable and repeatable", as discussed by Rear Admiral Paul Thomas:

[1:35:50] "If you look at the IMO guidelines on type approval of international systems, the standard is dead. The fact of the matter is that a number of administrations, because those guidelines are not mandatory, have approved systems that don't kill things. They apparently are satisfied with the efficacy tests. We have not been able yet to determine if efficacy tests are reliable and repeatable. We continue to look at that. There is an appeal. This is currently under review of the Coast Guard, we've got some new data. If we can determine that those tests are reliable and repeatable across a broad spectrum of species that you see in ballast water, then we will be in a better position to type approve those systems."

Slide 25 Notes

This concern is also reflected in the testimony of Rear Admiral Paul Thomas:

[1:37:36] "Intuitively you say that if I can render this organism so that it can never reproduce, that is effectively dead for the intent of the regulation. And to be quite honest, I would agree with that. The problem is demonstrating that you have in fact done that for every one of the organisms that might be in that ballast water. It is easier to demonstrate that they are dead than it is that they non-viable."

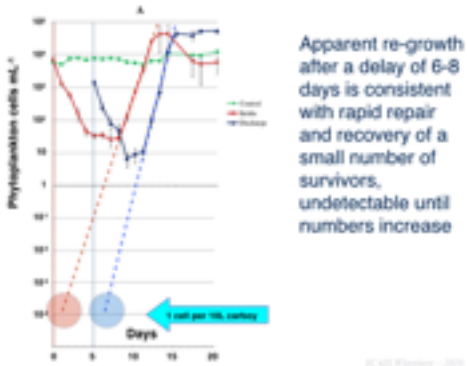
The following slides present a discussion of "re-growth" that is consistent with our publication, but adds a new illustration.

Slide 26 Notes

Repair of damage by ultraviolet radiation and "re-growth" is well known (Sinha and Häder 2002). A recent study by Liebich et al. (2012) is relevant. It was presented at a previous Conference on Aquatic Invasive Species.

Slide 27 Notes

These results from Liebich et al. (2012) show counts of total cells after initial treatment with ultraviolet radiation (Intake, red), and treated again after a 5-day hold (Discharge, blue). Each shows a decline and what the authors describe as re-growth after 7 days. The grow-out experiments were conducted in 10-liter carboys.

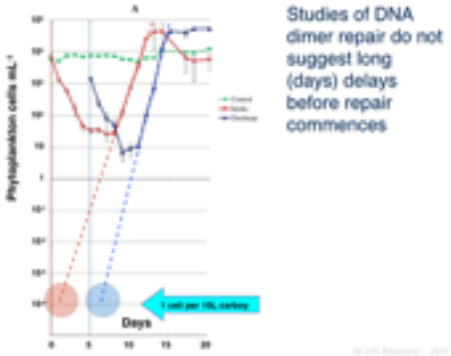


Slide 28 Notes

It is understandable to interpret these results as recovery of damaged phytoplankton after a period of about a week. There could be a concern about other phytoplankton that require even longer to recover. Many MPN tests are conducted over 14 days. What if cells recover too late to be detected by the method? (See slide 25)

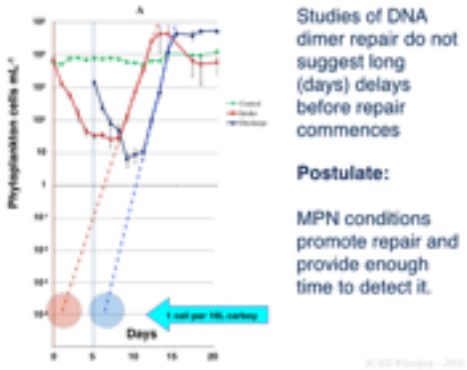
The authors of this study discussed their results very carefully in a well-written paper. Here we present one interpretation that they did not pursue in detail.

The graphs have been modified to show lower concentrations of cells (down to the concentration associated with one cell per 10-liter carboy) and hypothetical growth curves of viable cells, ultimately outnumbering the counts of total cells that decline as UV-treated, nonviable cells disappear over time.



Slide 29 Notes

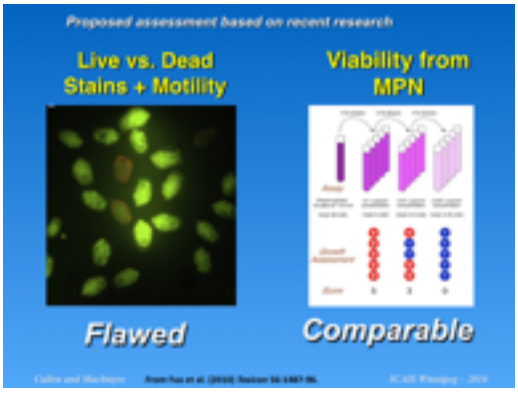
This scenario is consistent with what we know about repair of DNA damage. It is likely, although perhaps not conclusively demonstrated for all situations, that the repair occurs in hours to days, or not at all. We are not aware of evidence for delays of many days before the intracellular process of repair begins.



Slide 30 Notes

The apparent delay of a week before regrowth is consistent with relatively rapid repair and recovery that went undetected until viable cells grew enough to be detected.

As pointed out by Cullen and MacIntyre (2015) and also suggested by Liebich et al. (2012), the benign conditions in the MPN method would promote repair. We postulate that typical MPN tests of 14 days should provide enough time to detect phytoplankton that are capable of recovering from UV treatment.



Slide 31 Notes

We conclude that the assessment of the live-dead method vs. viability from MPN should be revised based on recent evidence.

In particular, recent peer-reviewed results of MacIntyre and Cullen (2016), comparing living vs. heat-killed phytoplankton, show that it is no longer scientifically justified to assume that the live/dead vital stains + motility method prescribed by the U.S. Coast Guard is reliable and repeatable for all species of phytoplankton. The FDA+CMFDA method is subject to considerable error, and for some species it cannot distinguish live from dead cells with any statistical confidence. Complementary observations of motility cannot help for species that are incapable of movement.

In turn, data and scientific arguments that are not tied to any particular treatment technology suggest that the MPN method is much less vulnerable to methodological uncertainties than has been commonly thought. The MPN method is subject to error, but these can be assessed and compared to those associated with stains + motility.