1   **TITLE:** Comparison of techniques used to count single-celled viable phytoplankton

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KEYWORDS

4   Phytoplankton; enumeration; FlowCAM®; flow cytometry; Sedgewick Rafter; ballast water; SYTOX Green,

5   CellTracker Green

ABSTRACT

6   Four methods commonly used to count phytoplankton were evaluated based upon the precision of concentration

7   estimates: Sedgewick Rafter and membrane filter direct counts, flow cytometry, and flow-based imaging cytometry

8   (FlowCAM). Counting methods were all able to estimate the cell concentrations, categorize cells into size classes,

9   and determine cell viability using fluorescent probes. These criteria are essential to determine whether discharged

10   ballast water complies with international standards that limit the concentration of viable planktonic organisms based

11   on size class. Samples containing unknown concentrations of live and UV-inactivated phytoflagellates (*Tetraselmis*

12   *impellucida*) were formulated to have low concentrations (<100 ml^{-1}) of viable phytoplankton. All count methods

13   used chlorophyll *a* fluorescence to detect cells and SYTOX fluorescence to detect non-viable cells. With the

14   exception of one sample, the methods generated live and non-viable cell counts that were significantly different

15   from each other, although estimates were generally within 100% of the ensemble mean of all subsamples from all

16   methods. Overall, percent coefficient of variation (CV) among sample replicates was lowest in membrane filtration

17   sample replicates, and CVs for all four counting methods were usually lower than 30% (although instances of ~60%

18   were observed). Since all four methods were generally appropriate for monitoring discharged ballast water,

19   ancillary considerations (e.g., ease of analysis, sample processing rate, sample size, etc.) become critical factors for

20   choosing the optimal phytoplankton counting method.

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INTRODUCTION

21   Photosynthetic plankton, or phytoplankton, are the foundation of the oceanic food web and are responsible

22   for approximately 50% of the global carbon fixation (Falkowski and Wilson 1992). Consequently, determining the

23   abundance, growth, and productivity of phytoplankton is crucial to understanding major oceanic biogeochemical

24   cycles and trophic pathways (Falkowski et al. 1998). Phytoplankton concentrations are frequently inferred from

25   bulk measurements, such as ocean surface color (Boyce et al. 2010), fluorescence (Welschmeyer 1994), or total

26   chlorophyll concentrations (e.g., Bidigare et al. 1986). These methods provide an overall assessment of the total

27   phytoplankton community, which includes various taxonomic groups (such as diatoms, dinoflagellates,

28   cyanobacteria) and ranges in size from picoplankton to macroalgae. Bulk measurements, however, do not address

29   some critical characteristics of the phytoplankton community, such as cell concentration, taxonomic composition, or

30   physiological status. While methods exist to characterize the community based upon photopigments (e.g., Mackey et

31   al. 1996), numerical counts of composite phytoplankton cannot be determined from bulk measurements. Relative

32   concentrations of chlorophyll *a* vary between algal taxa and can change in response to different environmental

33   conditions (Cloern et al. 1995; de Jonge and Colijn 1994). In algal monocultures, the ratio of chlorophyll *a* to cell

34   concentration changes with cell physiology and growth phase (Wirtz and Pahlow 2010). Therefore, bulk

35   measurements based upon chlorophyll *a* may be a poor proximal measurement of phytoplankton concentrations.

36   Instead, single cell counting methods, such as microscopy and flow cytometry, are required to precisely estimate

37   concentrations of phytoplankton (Lessard and Swift 1986; Veldhuis and Kraay 2000).

38   Microscopy has been used to examine chemically preserved samples collected on membrane filters

39   (Fahnenstiel et al. 1995) or settled in counting chambers (Willén 1976). Flow cytometry is well suited to detect

40   phytoplankton based on their natural chlorophyll fluorescence and was critical in the discovery of the superabundant

41   picoplankter *Prochlorococcus* and in advancing our understanding of the importance of picophytoplankton in

42   oceanic primary production (Chisholm et al. 1988). Instruments combining the imaging capability of microscopy

43   and the flow-through particle analysis of flow cytometry have also been used to count phytoplankton. Examples

44   include the FlowCAM® (Fluid Imaging Technologies, Brunswick, ME; Buskey and Hyatt 2006; See et al. 2005)

45   and the Flow Cytobot (BD, Franklin Lakes, NJ; Sosik et al. 2003; Olson and Sosik 2007). The novel imaging

46   cytometers have the shortest record of usage for counting phytoplankton, therefore, assessing the precision and

47 accuracy of these devices (relative to more established techniques) is valuable to set the optimal operating criteria

48 and detection limits.

49   In January 2008, a workshop was organized to evaluate four methods for enumerating viable

50 phytoplankton: flow cytometry, an enhanced flow-through system with imaging capacity (FlowCAM®), direct

51 counts of samples collected on membrane filters, and direct counts using a Sedgewick Rafter counting chamber. All

52 techniques used fluorescent stains to differentiate between live and dead cells. Counting methods were tested with

53 several ratios and densities of live and dead *Tetraselmis impellucida*, a small phytoflagellate. In the techniques

54 evaluated in this workshop, cell size was determined either from captured images, size references in the microscope

55 field, or light scattering signal for flow cytometry. Comparisons were conducted under ideal conditions with no

56 debris or particulate matter and with a single target species.

57   This work was conducted to guide the development of standard methods for counting planktonic organisms

58 $\geq$10 µm and < 50 µm in minimum dimension in studies of ballast water treatment. This size class is specified by the

59 International Maritime Organization (IMO) in the 2004 convention for managing ships' ballast water and sediments

60 that aims to reduce the spread of aquatic nuisance species via ballast water discharge. Standards set in the IMO

61 convention state that there should be less than 10 viable organisms $\geq$10 µm and < 50 µm in minimum dimension per

62 ml of water (IMO 2004). The proposed U.S. Phase I discharge standard is identical for this size class (Federal

63 Register 2009). Notably, it does not include all phytoplankton (because some phytoplankters are > 50 µm or < 10

64 µm in minimum dimension), nor is this size class exclusively composed of phytoplankton. Nevertheless,

65 phytoplankton are a dominant component of this size class in most aquatic systems, and, therefore, we evaluated the

66 four counting methods on their ability to detect and categorize phytoplankton based upon cell size and viability. The

67 IMO G8 guidelines define "viable organisms" as "organisms and any life stages thereof that are living" (2005); for

68 the purposes of this paper, organisms will be classified as either 'live' or 'dead'. Here we describe the precision of

69 the counting techniques and weigh the advantages and disadvantages of each for counting phytoplankton with

70 respect to ballast water issues.

METHODS

Sample preparation

71   To reduce the number of variables and provide a clear comparison between counting techniques,

72   concentrated monocultures of *Tetraselmis impellucida*. (strain PLY 429), an autotrophic flagellate, were shipped

73   from Reed Mariculture (Campbell, CA), which also provided an estimate of the cell density before packaging. The

74   mean cell width was approximately 10 μm. In addition to live cells, batches of *T. impellucida* were killed by

75   exposing them to UV light (a 122 cm, 30 Watt Ultra Violet Germicidal Lamp) for 30 minutes. The efficacy of this

76   UV treatment was verified by comparing concentrations of live and dead cells in treated and untreated samples

77   (described below). Samples 1- 6 were prepared by mixing live stock cultures with UV treated cultures at varying

78   ratios and diluting with room temperature artificial seawater (Instant Ocean®; Aquarium Systems, Inc., Mentor,

79   OH) for a total volume of 10 L, which was aliquoted to participants for analysis. The actual concentration of the

80   cultures could not be measured using any of the methods in the workshop without creating bias for that method, so

81   the densities of cells in the samples were estimated using the concentrations provided by the manufacturer. Fresh

82   sample concentrations with varying ratios of live and dead *Tetraselmis* were prepared over the course of six days for

83   analysis during the workshop and analyzed within 5 hours. Four methods of analysis were performed: flow

84   cytometry, direct counts on membrane filters, FlowCAM®, and direct optical counts in a Sedgewick Rafter counting

85   chamber.

Flow cytometry

86   Subsamples were analyzed using a Becton-Dickinson FACSort flow cytometer operated with CellQuest

87   Acquisition Software and CYTOWIN 4.31 analysis software. Using a flow rate of 60 µl min^{-1}, each run took

88   approximately 15 minutes to analyze 0.9 ml. Subsamples were incubated with the mortal stain SYTOX Green (0.5

89   µM, 15 min, Invitrogen, Life Technologies), which is cell impermeable and can only enter cells that have a

90   compromised membrane. When SYTOX Green binds to DNA, it has an excitation maximum of 504 nm and an

91   emission maximum of 523 nm. Cells were classified as live or dead based on their red chlorophyll fluorescence

92   without green DNA fluorescence or red chlorophyll fluorescence with green nuclear fluorescence, respectively.

93   Forward and side light scatter measurements were also used to help assess the target cell populations.

Direct counts on membrane filters

94   Subsamples (5 ml) were incubated at room temperature in the dark for 45 min after the addition of either

95   the "dead" vital stain, SYTOX Green (0.5 µM) or CellTracker™ Green CMFDA (5 µM, Invitrogen, Life

96   Technologies), an enzymatically activated "live" vital fluorescent stain. These subsamples were then preserved with

97   formalin (5% v/v) for 1 min to terminate the live cell enzymatic reaction with CMFDA, and then rinsed (3x with

98   filtered seawater, 3 ml each) and filtered onto 5-µm pore size, 25-mm diameter Whatman Cyclopore™

99   polycarbonate membrane filters and mounted on glass microscope slides with 25 µl glycerin to deter

100   photobleaching. Slides were enumerated at 200x magnification using a Lietz Diaplan microscope equipped with a

101   100-watt high-pressure mercury lamp and a blue light excitation filter set (480/40; 505; 510LP). For low density

102   samples, the entire filter was examined and counted, and for high-density samples, transects of the filter were

103   analyzed until 400 cells were tallied (Andersen and Throndsen 2003). Both living and dead cells had bright red

104   chlorophyll fluorescence that significantly aided in the identification of the *T. impellucida* cells. Live cells were

105   classified based on green fluorescence within the cytoplasm resulting from the CMFDA labeling, while dead

106   STYOX-stained cells had a distinct green fluorescent nucleus.

FlowCAM®

107   The FlowCAM® is a flow-through imaging cytometer (Fluid Imaging Technology, Yarmouth, ME). Algal

108   subsamples were stained with SYTOX Green (0.9 µM, 10 min) before being analyzed with the FlowCAM®, and a

109   stained blank (artificial seawater only) was used to calibrate the background fluorescence of SYTOX Green.

110   Subsamples (1 ml) were analyzed at a flow rate of 77 µl min^{-1}. An image of the triggered particle was automatically

111   taken when detected, and chlorophyll fluorescence, SYTOX Green fluorescence, and forward scatter intensities

112   were recorded. Images and measurements were then analyzed using VisualSpreadsheet software (Fluid Imaging) to

113   identify particles with only chlorophyll fluorescence (live cells) and particles with chlorophyll and SYTOX

114   fluorescence (dead cells). Cells that had no fluorescence of either spectrum were classified as 'unknown' and

115   included in the total cell counts.

Sedgewick-Rafter Counting Chamber

116   *Tetraselmis* cells were immobilized by adding two drops of acetic acid (Heinz vinegar) to 10 ml aliquots of

117   the sample. Subsamples (1 ml) of the immobilized stock culture were stained with SYTOX Green (0.9 µM),

118   incubated for 10 min in the dark, and transferred to a Sedgewick Rafter chamber etched with a 20 row x 50 column

119   grid. Chambers were examined with a Nikon E600 compound microscope with a blue excitation filter cube (470/40;

120   500; 515LP) at 100-200x magnification. For each subsample, ten rows were randomly selected and counted for a

121   final analysis volume of 500 µl. First, the total number of cells in a row was counted under brightfield illumination,

122   and then, the row was examined again under epifluorescence to identify the dead cells stained with SYTOX Green.

123   Live cells were calculated as the difference between total and dead cell counts.

Efficacy of UV treatment

124   Aliquots of *Tetraselmis* cells were inactivated with UV light before addition to samples. Twenty ml of

125   concentrated *Tetraselmis* culture ($\sim10^5$ cells ml^{-1}) were added to 10 cm diameter, 1.5 cm deep plastic Petri dishes.

126   Open dishes were placed approximately 13 cm below a UV germicidal lamp and incubated for 30 minutes. Control

127   samples were incubated in the same room outside of the safety cabinet and away from the UV light. Cultures were

128   kept at ambient conditions for 1 hour after the treatment to ensure damaged cells had time to die, at which point

129   CMFDA and fluorescein diacetate (FDA), another green, fluorescent "live" vital stain, were added to a subsample of

130   the cultures (5.0 and 2.5 µM, final concentrations, respectively). Stained samples were incubated for 10 min in the

131   dark and counted on Sedgewick Rafter counting chambers within 30 minutes of the start of the incubation. Total

132   cells were first counted under brightfield illumination; live cells were visualized and counted using epifluorecence

133   illumination. Dead cells were calculated as the difference between total and live cells.

Statistical Analysis

134   Live and total cell densities for each sample were first tested for homogeneity of variances with the

135   Levene's test (SPSS 13.0; Chicago, IL). If variances were statistically equal at the $p \leq 0.05$ confidence level, the

136   means were compared with ANOVA, and pair-wise comparisons were made with Tukey's HSD post-hoc test. If

137   variances were unequal, the means were compared with Welch's ANOVA, which assumes unequal variance, and

138   pair-wise comparisons were made with the Games-Howell post-hoc test. In addition, the coefficient of variation

139   (CV) was measured for the sample replicates of each method.

RESULTS AND DISCUSSION

140   The goal of this study was to assess four different techniques used to determine phytoplankton

141   concentrations and viability. The techniques were chosen based upon several criteria. First, the techniques should

142   be able to count the number of individual cells in a suspension. Second, the techniques should be able to measure

143   the dimensions of the organisms. Cells measurement data were not collected when the techniques were used in the

144   study presented here; however, determining if cells are within a specific size class may be critical for certain studies,

145   such as evaluating the efficacy of treatment for ballast tank discharge water. Finally, the methods must be capable

146   of distinguishing between live and dead phytoplankton, and techniques must allow for rapid processing to ensure

147   that live cells do not die before they are analyzed. The reader is directed to another comparative study of

148   phytoplankton counting methods by Karlson et al (2010). A number of different methods were compared for total

149   cell counts, but no studies were made on cell viability.

150   Because the actual stock culture concentration was unknown, an 'ensemble' mean was calculated by

151   averaging the concentrations measured by each method. If one of the methods (e.g. Sedgewick Rafter counting

152   chambers) had been used to determine the initial concentration and percent viability of *Tetraselmis* in culture, then it

153   is possible that the comparisons of the methods would have been biased to the method chosen to initially measure

154   culture concentrations. Although Sedgewick Rafter chambers are known to be very accurate with high densities of

155   cells, the samples used in this study had low organism densities to simulate treated ballast water. The disadvantage

156   of using an ensemble mean is its sensitivity to extreme measurements. Also, this approach implies that the true

157   concentrations fall within the range of concentrations measured by the different techniques. Nevertheless, the

158   difference in measured cell concentrations among methods was never more than a factor of two, though they were

159   significantly different from each other. Total cell concentrations in the test samples were <1000 ml^{-1} and typically

160   less than 100 ml^{-1}, falling within the range of phytoplankton concentrations observed in some near-shore

161   environments (Olson and Sosik 2007) but well below concentrations observed during bloom conditions where

162   phytoplankton density can approach 10^6 cells ml^{-1} (e.g., Buskey et al. 2001).

163   Methods used to detect phytoplankton in treated ballast water discharge must be able to detect live

164   phytoplankton amid high concentrations of dead cells. In this study, UV light was used to kill cultured cells. In a

165   subset of trials designed to validate our method of killing cells, live *Tetraselmis* in a positive control culture

166   comprised approximately 88 ± 12 % of the total cell count whereas live *Tetraselmis* accounted for only 0.3 ± 0.3%

167   of the total cell count in UV-treated samples. Therefore, UV treatment was sufficient to kill > 99% of the cells and

168 significantly reduced the concentrations of live cells relative to control treatments (ANOVA, $p < 0.05$; data not

169 shown). Viability in these trials was tested with the "live" vital stains CMFDA and FDA, which react with cytosolic

170 enzymes. Therefore, the UV treatment either deactivated the enzymes involved in transforming the fluorochromes or

171 prevented intracellular accumulation of the stains within cells.

172   A general approach for classifying live and dead cells was to count total phytoplankton (using chlorophyll

173 *a* fluorescence to identify cells) and dead phytoplankton (using SYTOX fluorescence); live cell concentrations were

174 the difference between total and dead counts. SYTOX has previously been used to label phytoplankton with

175 compromised cell membranes (Brussaard et al. 2001), but has been found to underestimate the relative portion of

176 dead cells in certain cases (e.g., when cells have damaged DNA; Lebaron et al. 1998). An alternative to counting

177 dead cells is to use "live" vital stains (e.g. CMFDA and FDA) to directly count living cells. Only one method in this

178 study used a live vital stain.

179   With the exception of Sample 4, the concentrations of live cells measured by each method were

180 significantly different within each sample (ANOVA, $p \leq 0.05$; Figure 1). The concentrations of total cells measured

181 by each method were also significantly different except for Sample 1 ($p = 0.06$). The FlowCAM® method

182 generated cells classified as "unknown" (i.e., cells which had neither chlorophyll fluorescence nor SYTOX Green

183 fluorescence), and these objects were included in the total cell counts. The percentage of indistinguishable cells

184 varied for each sample; in Sample 1, the indistinguishable cell concentration was 26 cells ml^{-1}, or approximately

185 53% of the total count. However, in samples 4, 5, and 6, indistinguishable cells represented less than 10% of the

186 total cell concentration.

187   Samples contained a range of total cell concentrations (24 to 880 cells ml^{-1}) and percentages of living cells

188 (12 – 63%). There was no significant relationship between the percent difference from the ensemble mean and the

189 concentrations measured by each method (Figure 2); however, some trends were observed. Flow cytometry

190 concentrations were typically less than ensemble means, whereas membrane filtration concentrations were typically

191 above the ensemble means. The precision of different methods was measured by the percent coefficient of variation

192 (CV) between sample replicates. Membrane filtration and direct counting showed the lowest mean CV (Table 2).

193 The highest CVs were calculated from the ensemble means, demonstrating that the variation among sample methods

194 was greater than sample variations among subsamples analyzed by the various methods used in this workshop.

195   In this study, the cell-enumeration techniques were primarily evaluated by precision of estimated

196 phytoplankton concentrations. However, other factors should be considered when using these techniques for other

197 purposes: sample volume and sample analysis rate, ease of use, and documentation of results. The FlowCAM® has

198 the advantage of documenting (by collecting an image of) all objects passing through the flow cell (Poulton and

199 Martin 2010), and objects with similar properties (e.g. circularity, length, etc.) can be categorized. The FlowCAM®

200 also acts as particle counter and collects light scatter and fluorescence intensity signals similar to a flow cytometer.

201 Interchangeable flow cells allow for the use of different objective lenses and magnifications. However, changing

202 the flow cell likely changes the fluorescence and light scattering measurements. The flow cells generally allow a

203 higher rate of sample analysis than flow cytometers (approximately 80 µl min^{-1} vs. 60 µl min^{-1}, respectively).

204 Without the use of sheath fluid to hydrodynamically focus the sample stream (as in flow cytometry), some imaged

205 particles were out of focus, and the unfocused sample stream also likely contributes to variability in light scattering.

206 This method was the only method tested that labeled cells as "unknown" in addition to "live" and "dead" and will

207 likely have trouble identifying cells with generic, non-descript features.

208   Using flow cytometry, samples were analyzed at approximately 60 µl min^{-1}. Particles passing through the

209 interrogation point are hydrodynamically focused and, therefore, variations in the light scattering signals and

210 fluorescence intensity should not vary due to the location of the particle in the flow cell (as may be the case for the

211 FlowCAM®). However, the actual size of particles is not measured directly. Often, calibrated microbeads with a

212 known diameter are used to roughly approximate size based upon the light scattering signals. This approach

213 provides an estimate of particle size but cannot verify the dimension of the object, much less other properties such as

214 circularity or aspect ratios. As such, it will be difficult to identify different species in a mixed assemblage using

215 flow cytometry as opposed to FlowCAM®, which images each organism. Furthermore, with both FlowCAM® and

216 flow cytometry, a sample reservoir is used to feed the sample into the fluidics system. There is a potential that, over

217 long sample analysis periods (required for large sample volumes to reduce counting errors with low density

218 samples), non-neutrally buoyant particles or swimming cells will sink or float. This process can lead to incorrect

219 estimates of cell concentrations as swimming cells and particles fractionate in the sample reservoir. Additionally, it

220 may be difficult to processes a sufficient volume of sample before live cells begin to die due to handling stress.

221   Both Sedgewick-Rafter counting chambers (McAlice 1971) and combining membrane filtration with

222 epifluorecence microscopy (Hobbie et al. 1977) have been used for decades for counting planktonic organisms, and

223 the operating parameters and limitations for these methods have been well documented. For example, uneven

224 distributions of cells either in the counting chamber or on the membrane filter generate inaccurate results (Andersen

225 and Throndsen 2003). Also, the fading of the fluorescence signal (i.e., photobleaching) can occur as samples are

226 exposed to light. Another limitation is the longer sample analysis time and lower volumes of sample that can be

227 analyzed. This is especially true with Sedgewick Rafter counting chambers, where the maximum sample volume is

228 1 ml and the limit of detection is 1000 cells L^{-1} if no concentration steps are employed (LeGresley and McDermott

229 2010). The sample volume can be adjusted for membrane filtration and higher volumes can be filtered to detect

230 sparse populations (Booth 1993). Furthermore, the filter pore size can be selected to target specific size classes (e.g.

231 > 10 µm). One disadvantage of membrane filtration is that cells cannot be viewed with brightfield illumination as

232 the membrane filter distorts the image, and identifying different species and taxa would be difficult. The sample

233 processing rate for manual microscopy, therefore, is highly dependent upon the volume filtered, the percentage of

234 the chamber or filter surveyed, and other factors such as the cell concentration and the amount of debris.

235 Additionally, manual microscopy allows for classifying and imaging individual organisms; however, identifying

236 specific species generally requires a high level of taxonomic skill (Karlson et al. 2010).

237 Although concentration estimates from the methods evaluated in this workshop were within 2x of each

238 other, a fairly high variation among methods was observed. In some cases, the variation could affect whether the

239 sample meets the IMO discharge standard of < 10 cells ml^{-1}. For example, in Sample 4, flow cytometry measured 8

240 viable cells ml^{-1}; other methods measured > 20 cells ml^{-1}. For techniques used to detect sparse populations from

241 treated ballast water discharge, it is important to validate that the method is sensitive enough to detect low densities

242 of viable organisms. Also, as seen from the variable results between samples, it may be most efficient to perfect a

243 single technique for the purpose of testing treated ballast water in order to increase the precision, and most

244 importantly, techniques must be validated using complex assemblages of natural plankton.

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REFERENCES

253   Andersen P, Throndsen J (2003) Estimating cell numbers. In: Hallegraeff GM, Anderson DM, Cembella AD (ed)
254   Manual on Harmful Marine Microalgae. Paris, UNESCO, pp 99-129

255   Bidigare RR, Frank TJ, Zastrow C, Brooks JM (1986) The distribution of algal chlorophylls and their degradation
256   products in the Southern Ocean. Deep Sea Res Part A. Oceanogr Res Papers 33: 923–937

257   Booth BC (1993) Estimating cell concentration and biomass of autotrophic plankton using microscopy. In: Kemp
258   PF, Sherr BF, Sherr EB, Cole JJ (ed) Handbook of Methods in Aquatic Microbial Ecology. Boca Raton,
259   Lewis Publishers, pp. 199–205

260   Boyce DG, Lewis MR, Worm B (2010) Global phytoplankton decline over the past century. Nature 466: 591–596

261   Brussaard CP, Marie D, Thyrhaug R, Bratbak G (2001) Flow cytometric analysis of phytoplankton viability
262   following viral infection. Aquatic Microbial Ecol 26: 157–166

263   Buskey E J, Hyatt CJ (2006) Use of the FlowCAM for semi-automated recognition and enumeration of red tide cells
264   (Karenia brevis) in natural plankton samples. Harmful Algae 5: 685–692

265   Buskey E, Liu H, Collumb C, Bersano J (2001) The decline and recovery of a persistent Texas brown tide algal
266   bloom in the Laguna Madre (Texas, USA). Estuaries Coasts 24: 337–346

267   Chisholm SW, Olson RJ, Zettler ER, Goericke R, Waterbury JB, Welschmeyer NA (1988) A novel free-living
268   Prochlorophyte abundant in the oceanic euphotic zone. Nature 334: 340–343

269   Cloern JE, Grenz C, Vidergar Lucas L (1995) An empirical model of the phytoplankton chlorophyll:carbon ratio:
270   the conversion factor between productivity and growth rate. Limnol Ocean 40: 1313–1321

271   de Jonge VN, Colijn F (1994) Dynamics of microphytobenthos biomass in the Ems estuary. Mar Ecol Prog Ser 104:
272   185–196

273   Fahnenstiel GL, McCormick MJ, Lang GA, Redalje DG, Lohrenz SE, Markowitz M, Wagoner B, Carrick HJ (1995)
274   Taxon-specific growth and loss rates for dominant phytoplankton populations from the northern Gulf of
275   Mexico. Mar Ecol Prog Ser 117: 229–239

276   Falkowski PG, Wilson C (1992) Phytoplankton productivity in the North Pacific ocean since 1900 and implications
277   for absorption of anthropogenic CO_2. Nature 358: 741–743

278   Falkowski PG, Barber RT, Smetacek V (1998) Biogeochemical controls and feedbacks on ocean primary
279   production. Science 281: 200–206

280   Federal Register (2009) Standards for Living Organisms in Ships' Ballast Water Discharged in U.S. Waters; Draft
281   Programmatic Environmental Impact Statement, Proposed Rule and Notice, 74 FR 44631-44672 (28
282   August 2009). National Archives and Records Administration, Washington, DC.

283   Hobbie J, Daley R, Jasper S (1977) Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl
284   Env Microbiol 33: 1225–1228

285   Karlson B, Cusack C, Bresnan E (2010) Microscopic and molecular methods for quantitative phytoplankton
286   analysis. Paris, UNESCO. (IOC Manuals and Guides, no. 55.) (IOC/2010/MG/55)

287   LeGresley M, McDermott G (2010) Counting chamber methods for quantitative phytoplankton analysis -
288   haemocytometer, Palmer-Maloney cell and Sedgewick-Rafter cell. In: Karlson B, Cusack C, Bresnan E
289   (ed) Microscopic and molecular methods for quantitative phytoplankton analysis. Paris, UNESCO. (IOC
290   Manuals and Guides, no. 55.) (IOC/2010/MG/55)

291   International Maritime Organization (2004). International convention for the control and management of ships'
292   ballast water and sediments. http://www.imo.org/conventions/mainframe.asp?topic_id=867. Accessed 01
293   October 2010.

294   Lebaron P, Catala P, Parthuisot N (1998) Effectiveness of SYTOX green stain for bacterial viability assessment.
295   App Env Microbiol 64: 2697–2700

296   Lessard EJ, Swift E (1986) Dinoflagellates from the North Atlantic classified as phototrophic or heterotrophic by
297   epifluorescence microscopy. J Plankton Res 8: 1209-1215

298   Mackey MD, Mackey DJ, Higgins HW, Wright SW (1996) CHEMTAX - A program for estimating class
299   abundances from chemical markers: Application to HPLC measurements of phytoplankton. Mar Ecol Prog
300   Ser 144: 265–283

301   McAlice BJ (1971). Phytoplankton sampling with Sedgwick-Rafter cell. Limnol Ocean 16: 19–28

302   Olson RJ, Sosik HM (2007) A submersible imaging-in-flow instrument to analyze nano-and microplankton:
303   Imaging FlowCytobot. Limnol Ocean: Methods 5: 195–203

304   Poulton NJ, Martin JL (2010) Imaging flow cytometry for quantitative phytoplankton analysis - FlowCAM. In:
305   Karlson B, Cusack C, Bresnan E (ed) Microscopic and molecular methods for quantitative phytoplankton
306   analysis. Paris, UNESCO. (IOC Manuals and Guides, no. 55.) (IOC/2010/MG/55)

307   See JH, Campbell L, Richardson TL, Pinckney JL, Shen R, Guinasso NL (2005) Combining new technologies for
308   determination of phytoplankton community structure in the northern Gulf of Mexico. J Phycol 41: 305–310

309   Sosik HM, Olson RJ, Neubert MG, Shalapyonok A, Solow AR (2003) Growth rates of coastal phytoplankton from
310   time-series measurements with a submersible flow cytometer. Limnol Ocean 48: 1756-1765

311   Veldhuis MJ, Kraay GW (2000) Application of flow cytometry in marine phytoplankton research: current
312   applications and future perspectives. Sci Mar 64: 121-134

313   Welschmeyer NA (1994) Fluorometric analysis of chlorophyll-a in the presence of chlorophyll-b and pheopigments.
314   Limnol Ocean 39: 1985–1992

315   Willén E (1976) A simplified method of phytoplankton counting. British Phycological Journal 11: 265-278

316   Wirtz KW, Pahlow M (2010) Dynamic chlorophyll and nitrogen: carbon regulation in algae optimizes instantaneous
317   growth rate. Mar Ecol Prog Ser 402: 81–96

318

FIGURE CAPTIONS

319 **Figure 1.** *Tetraselmis* concentrations measured in six test samples using the counting techniques evaluated in the

320 workshop: flow cytometry, membrane filtration ('Filter'), imaging flow cytometry (FlowCAM®), and

321 Sedgewick Rafter counting chambers using SYTOX. Mean live and dead *Tetraselmis* concentrations are

322 shown with standard deviations. The red dashed line represents the ensemble mean of total cell abundance,

323 and the black dashed line is the ensemble mean of live cell abundance. FlowCAM® analysis included cells

324 that were not distinguished as live or dead. These cells are classified as unknown.

325

326 **Figure 2.** Percent difference from the ensemble sample for all six samples measured using the counting techniques

327 evaluated in this workshop. Differences from the live and dead ensemble sample are shown in the top and

328 bottom panels, respectively.

329

330 **Figure 3.** Coefficient of variation (% CV) measured by each method for all six samples compared to mean

331 *Tetraselmis* concentration. Live and dead concentrations determined using the counting techniques in the

332 workshop and the ensemble mean are shown in the top and bottom panels, respectively.
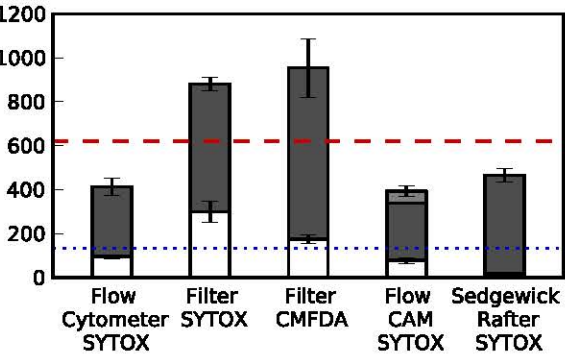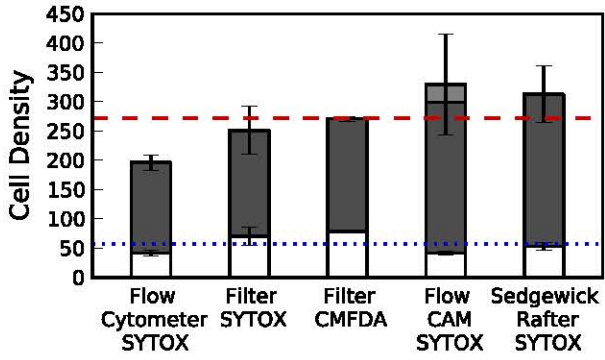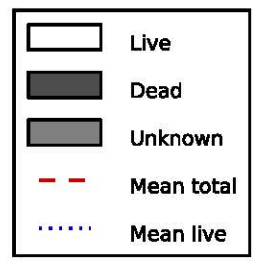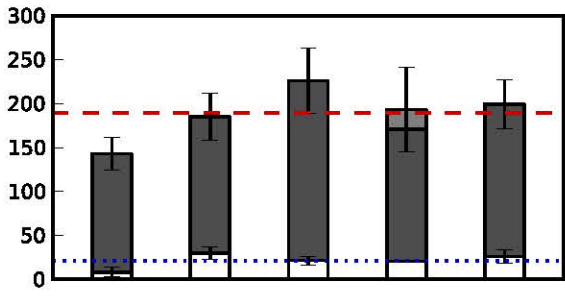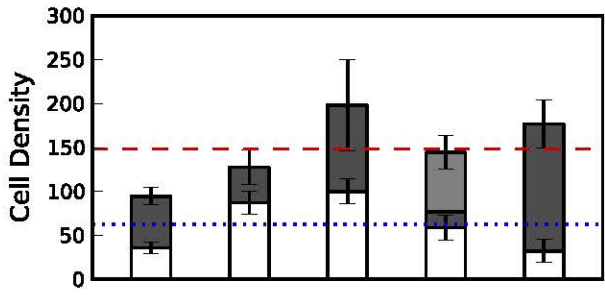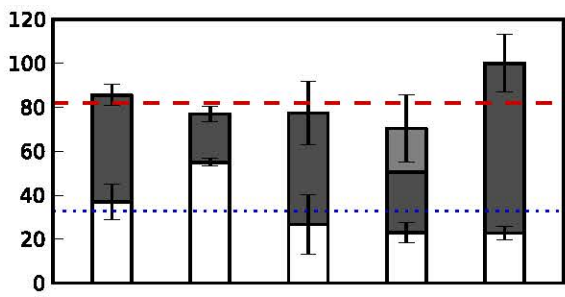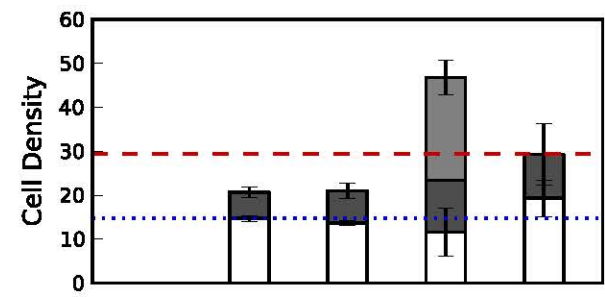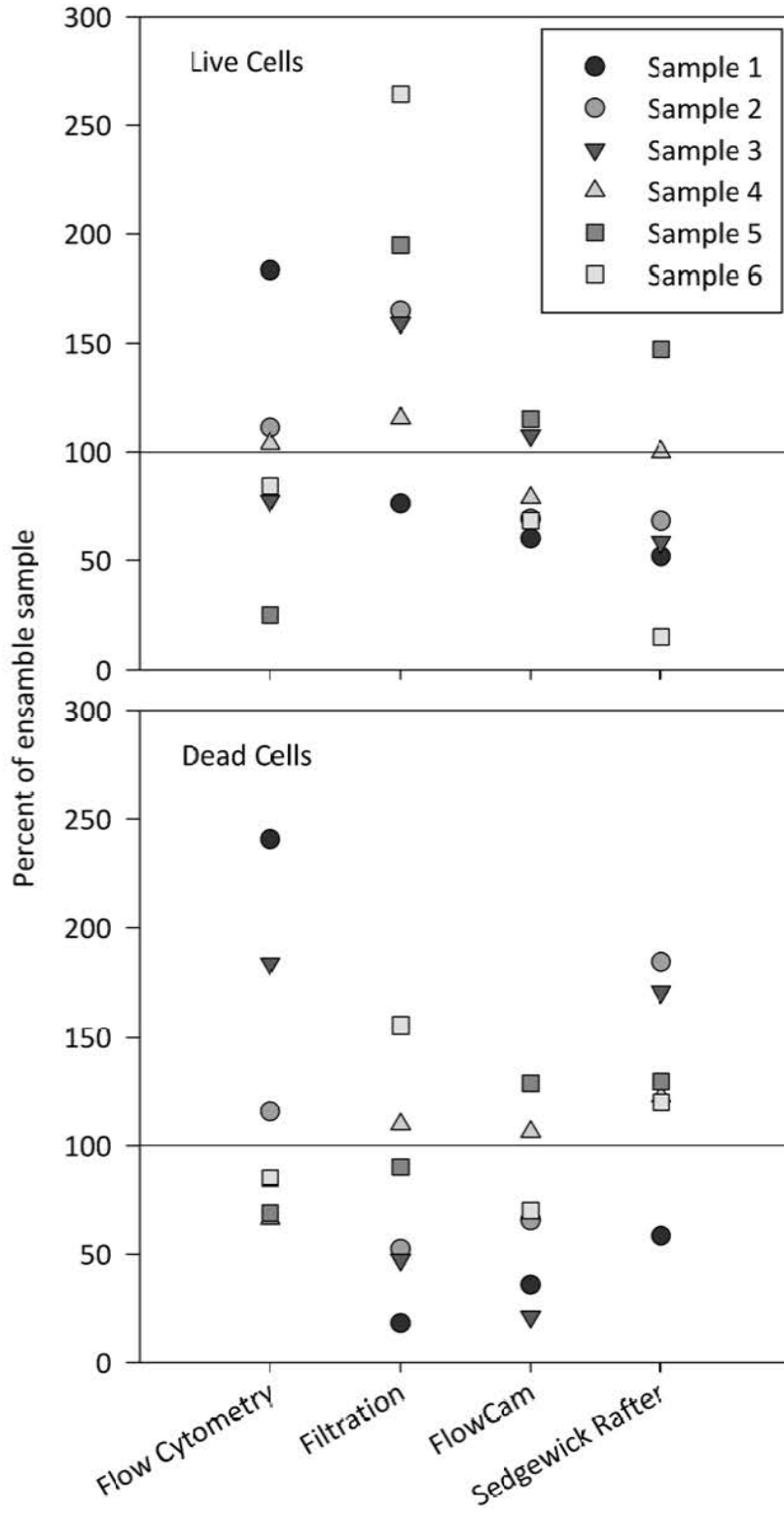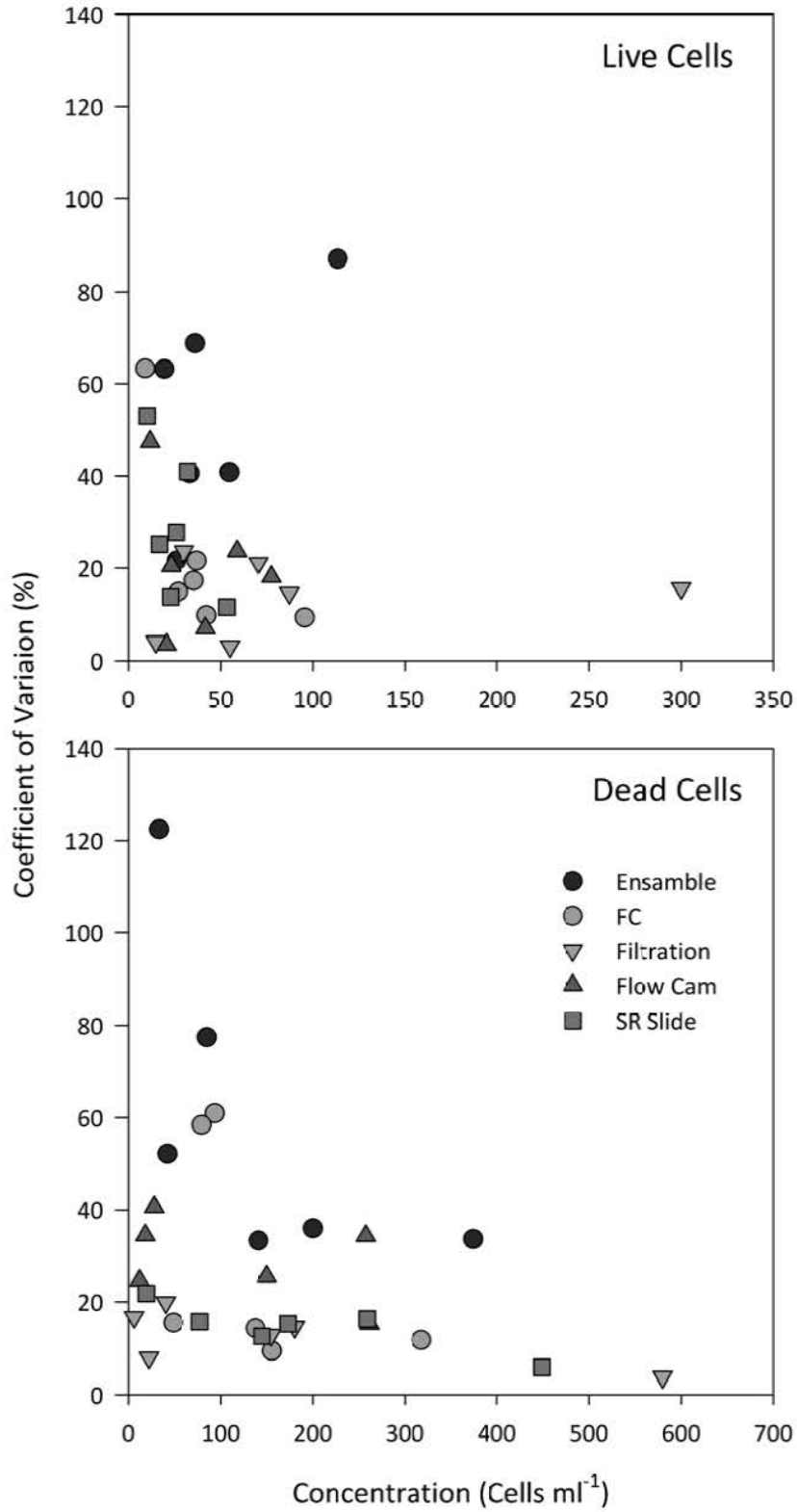
Table 1. Estimated cell densities for each method compared to the sample means. The number of subsamples analyzed by each method ranged from 2-6. Samples are listed in order of increasing total cell concentration (cells ml^{-1}).

Sample	Ensemble Mean Live	Ensemble Mean Total	% Live	Flow Cytometry SYTOX Live	Flow Cytometry SYTOX Total	Filtration SYTOX Live	Filtration SYTOX Total	Filtration CMFDA Live	Filtration CMFDA Total	FlowCAM® Live	FlowCAM® Total	Sedgewick-Rafter Live	Sedgewick-Rafter Total
1	15	24	63%			15	21	14	21	12	50	19	29
2	33	83	40%	37	86	55	77	27	77	23	70	23	77
3	63	135	47%	36	95	87	128	100	198	59	144	32	177
4	21	186	12%	8	143	30	185	22	201	21	193	26	199
5	57	266	21%	42	196	71	251	79	259	42	329	53	313
6	126	580	22%	96	414	300	880	174	955	78	394	17	465

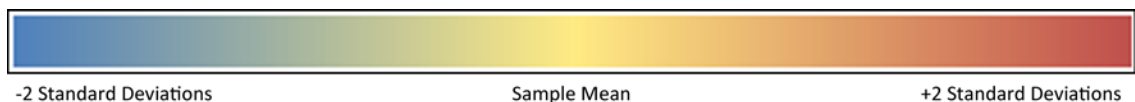-2 Standard Deviations   Sample Mean   +2 Standard Deviations

Table 2. Coefficient of variation (% CV) for all six samples analyzed using the methods evaluated in this workshop: flow cytometry, membrane filtration, FlowCAM®, and Sedgewick Rafter counting chambers. The ensemble mean was calculated from the concentrations measured by each method.

	Live Mean CV (±SD)	Live Min CV	Live Max CV	Dead Mean CV (±SD)	Dead Min CV	Dead Max CV
Ensemble	54 ± 24	22%	87%	59 ± 35	33%	122%
FC	23 ± 20	9%	63%	28 ± 24	9%	61%
Filtration	14 ± 8	3%	24%	13 ± 6	4%	20%
FlowCAM®	20 ± 16	3%	47%	29 ± 9	15%	41%
SR Slide	29 ± 16	11%	53%	15 ± 5	6%	22%