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NOTE

Temperature-induced microbubbles within natural marine samples may inflate small-particle counts in a Coulter Counter

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ABSTRACT: The Coulter Counter, a common instrument used to enumerate phytoplankton, may over-estimate counts of particles <2.5 µm in equivalent spherical diameter (ESD) by an order of magnitude when samples are run at temperatures cooler than ambient laboratory conditions. This phenomenon is likely due to microbubbles generated as a colder sample warms. Evidence for this mechanism derives from the observation that increasing the relative fraction of organic-rich coastal water in warming samples results in increased amplification of small-particle counts due to the stabilization of microbubbles. Count amplification can be eliminated by ensuring there is no temperature difference between the diluent and the sample. Failing to correct for this error confounds analysis of marine phytoplankton size spectra, complicating a broad range of experiments from those measuring productivity to those used to develop ecosystem-based models.

KEY WORDS: Coulter Counter · Microbubbles · Temperature · Particle size · Picoplankton

INTRODUCTION

The observation that transient bubbles <5 µm in diameter exist in natural seawater (NSW) was first made by Medwin (1977), and developed by Cooke & Johnson (1981), who noted that surface films of colloidal organic matter stabilize these 'microbubbles.' The medical imaging field makes extensive use of stabilized microbubbles to improve ultrasound contrast, and frequently employs a Coulter Counter to verify their presence (Takalkar et al. 2004, Chatterjee et al. 2005, Patil et al. 2009). Warming of a solution containing organic compounds and surfactants has also been confirmed to generate stabilized microbubbles by Lundgren et al. (2006), yet the possibility of such microbubbles occurring in cold samples of NSW or phytoplankton as they warm rapidly while in contact with diluent in a Coulter Counter has not yet been addressed. Since the significant contribution of the 1–2 µm phytoplankton size class to photosynthesis and the flux of organic matter in oligotrophic marine systems is becoming more and more evident (e.g. Richardson & Jackson 2007, Lomas & Moran 2010), accurately quantifying the dynamics of this phytoplankton size class is critical to our understanding of marine ecosystems.

The Coulter Counter is commonly used by marine ecologists and generally regarded as a highly robust method for counting and sizing biotic and abiotic particles (Baretta & Malschaert 1985). Coulter Counters have recently been employed to identify, count, and size phytoplankton in continuous culture experiments (Cermeño et al. 2011), mesocosm-enrichment experiments (Engel et al. 2008), and classical batch-culture experiments (Franklin et al. 2010), and to characterize particle-flux in the coastal environment

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(Reynolds et al. 2010). In addition, granulometric characteristics of marine abiotic particles are measured by sedimentologists using Coulter Counters (e.g. Sondi & Juracic 2010). However, our experiments indicate that modification of the current methodology is necessary to accurately quantify particle size distribution in samples of different temperatures.

The Coulter Counter uses size-calibrated interruptions across an aperture with a field of known voltage to count and estimate the diameter (equivalent spherical diameter, ESD) of particles passing through the aperture. The operating temperature range is 5 to 40°C according to the manufacturer, and thus the instrument should be expected to give accurate results within this temperature range. Field-based applications of the Coulter Counter have been conducted year-round with temperature conditions within the reported operating parameters of the device (Mulligan & Kingsbury 1968, MacDonald & Thompson 1985, Navarro & Thompson 1995). Mesocosm-based studies of particle size-spectra have also been conducted at a range of ambient temperatures (Li & Logan 1995). Previous laboratory studies of particle size and concentrations reference the temperature at which the samples were incubated (Hargis 1977) or obtained (Jackson et al. 1997), but rarely reference the temperature of the sample during analysis. Earlier papers reviewing the efficacy of the Coulter Counter focused on errors related to conductivity and coincidence (Edmundson 1966, Kersting 1985) and cell growth or breakage (Hastings et al. 1962, Baretta & Malschaert 1985). We believe the present study is the first to demonstrate that temperature can also be an important source of counting error, especially for small phytoplankton cells.

In the present study we demonstrate that the formation of microbubbles due to temperature disparities across the Coulter Counter aperture may falsely enhance counts of particles of <2.5 µm ESD by several orders of magnitude. This effect is strongest in NSW samples with complex organic matrices, and increases with the fraction of NSW relative to 0.22 µm Millipore Isopore™ membrane-filtered Instant Ocean® (FIO). It is recommended that researchers be aware of this complication when utilizing this type of instrument for picoplankton or small-particle counting.

MATERIALS AND METHODS

All experiments described here were conducted on a Coulter Counter Multisizer™ 3 in the Stewart Marine Biogeochemistry Laboratory at Queens College, City University of New York (CUNY). The proper functioning of the machine was confirmed by a Beckman Coulter representative before and after these experiments. In all experiments, a 50 µm aperture was used and calibrated with Beckman Coulter 5, 10, and 20 µm polystyrene beads. For all experiments, the diluent was particle-free FIO with salinity 35, the sample volume was 100 µl, and the sample flow rate across the aperture was 26.60 µl s^{-1}. During experiments where cooled diluent was used, the current and gain on the instrument were re-set following manufacturer instructions. All experiments were run with 4 to 5 replicates of each treatment. To verify the counting accuracy of our Coulter Counter Multisizer 3, replicate suspensions of 20 µm polystyrene calibration beads in FIO were counted by the instrument at room temperature. From these results, the number of beads in 0.9 µl (the volume of a hemacytometer chamber) were calculated and compared to counts under the microscope using a Neubauer hemacytometer (50× magnification). Replicate suspensions of beads were then counted using the hemacytometer in order to estimate the concentration in 100 µl (the volume of our Coulter Counter sample), and aliquots of the same solution were run on the Coulter Counter. Neither the hemacytometer estimate nor Coulter Counter estimates were significantly different from results obtained with the other method ($\alpha = 0.05$; Table 1).

Experiments were conducted to establish the influence of temperature on the Multisizer's reported size spectra of particles in natural samples. Local water from Gardiner's Bay in Long Island Sound (NSW of salinity 35) was filtered using a 0.22 µm Isopore filter to remove particles. To test whether cooler temperatures on their own or a temperature difference between the diluents and the sample affected size spectra, 2 sets of salinity-35 treatments (Series A and B) were prepared from a 1:10 mixture of 100 ml of filtered coastal water and 900 ml FIO. Since FIO contains less dissolved organic matter (DOM) relative to coastal seawater (Dai & Benitez-Nelson 2001), its role was to dilute DOM in a controlled manner. In Series A, the particle size spectra of the samples were analyzed with both the sample and diluent at the same temperature; counts were performed with both liquids at 20°C, then 10°C, and finally equilibrated back to 20°C. In Series B, the diluent remained at 20°C, while the sample began at 20°C, was cooled to 10°C, and finally was re-warmed to 20°C. Cooling of samples and diluents was done in a 10°C incubator, while equilibration to room temperature was achieved outside the incubator. All temperatures were confirmed with a digital thermometer.

Table 1. (A) Replicate samples of 20 µm polystyrene beads suspended in 0.22 µm filtered Instant Ocean artificial seawater were run on the Coulter Counter at room temperature. From these results, the number of beads expected in 0.9 µl of sample (the volume of the Neubauer hemacytometer) were calculated. This concentration was verified via microscopy in the hemacytometer. (B) In the reverse experiment, replicate samples of beads were prepared in the same manner as before but the hemacytometer was used to estimate the concentration in 100 µl, and this count was confirmed on the Coulter Counter. Counts from the hemacytometer estimate were not significantly different from the Coulter Counter reading ($p = 0.7279$ at $\alpha = 0.05$)

(A) Sample no.	Coulter Counter counts (cells per 0.9 µl)	Hemacytometer counts (cells per 0.9 µl)
1	8.84	8.6
2	7.62	9.2
3	10.02	11.2
4	11.26	11
5	3.03	4
6	3.39	4
7	3.36	3
8	2.83	3.33
Mean ± SE	6.29 ± 1.24	6.79 ± 1.25
(B) Sample no.	**Hemacytometer counts (cells per 100 µl)**	**Coulter Counter counts (cells per 100 µl)**
1	118.89	114.28
2	91.11	112.32
3	133.33	104.92
4	113.33	107.54
5	144.44	108.96
6	97.78	108.14
Mean ± SE	111.48 ± 8.31	109.36 ± 1.39

Experiments were also performed with monocultures of small (<2 µm) cells to determine the degree of amplification in laboratory-cultured samples and impact on cell counts. Batch axenic monocultures obtained from Bigelow Marine Laboratories of *Synechococcus bacillaris* (CCMP 1333) (1–2 µm cell size) were grown under sterile conditions in f/2 media prepared with FIO at a salinity of 35 (Guillard & Ryther 1962). Cultures were maintained at a temperature of 15°C in a 14:10 h light:dark cycle. *S. bacillaris* cells were counted on the Multisizer 3 at room temperature (20°C) and after cooling in the incubator (10°C), while the diluent temperature remained constant (20°C) using the procedure described above.

To determine if amplification was due to DOM, experiments were conducted with 0.22 µm filtered NSW and FIO, both at salinity 35. This approach was chosen since coastal seawater is relatively rich in DOM (Mannino & Harvey 2000), while minimal organic matter is present in 0.22 µm FIO (Dai & Benitez-Nelson 2001). The Connecticut Department of Energy and Environmental Protection (CT DEEP) measures the amount of dissolved organic carbon (DOC) monthly in coastal water, and the typical range is 1 to 4 ppm (CT DEEP 2010). The DOC content of the Nanopure water used to make FIO was confirmed to be below the 4 ppb detection limit of a Shimadzu TOC/TON analyzer (data not shown), and hence we are confident that it was properly diluting the DOM in the NSW. Samples consisted of FIO alone, NSW diluted 20 times with FIO, NSW diluted 5 times with FIO, and undiluted NSW. In these experiments, 4 replicates of each water type were counted under the following treatments: 20°C diluent/20°C sample, 20°C diluent/10°C sample, and 20°C diluent/20°C sample again. In this and all previous experiments, control blanks of FIO were run between samples to ensure samples were not cross-contaminated. Control blanks never varied >10%.

RESULTS AND DISCUSSION

In the first experiment, a paired Student's *t*-test revealed no significant difference between counts for any of the samples in Series A ($p = 0.07, 0.45, 0.84$ at $\alpha = 0.05$), indicating that the elimination of the temperature contrast between sample and diluent eliminates amplification in 1–2 µm counts. In Series B, samples 10°C cooler than the diluent (at 20°C) had 1800% higher counts in the 1–2 µm range relative to initial 1–2 µm counts when sample and diluent were at the same temperature (20°C) (Fig. 1). After the samples were equilibrated back to room temperature, average 1–2 µm counts for Series B went from 8.22×10^4 (±664 SE) during the cooled run to 3199 particles (±234 SE), significantly lower than the initial mean count of 4178 (±211) 1–2 µm particles (Fig. 1; $p = 0.01$ at $\alpha = 0.05$). These results suggest that amplification of the counts in the 1–2 µm particle range occurred only when the 10°C sample was in contact with the 20°C diluent.

When cooled to at least 10°C below the temperature of the diluent, samples containing a suspension of *Synechococcus bacillaris* displayed a 2235% (±56.8 SE) increase in particle counts in the size range of 1–2 µm relative to the counts in this range when the samples were run at 20°C (Fig. 2). We do not believe that all of this increase in cell counts could be due to rapid cell growth or lysis, since the counts decreased an order of magnitude when rewarmed to room temperature (Fig. 2). Furthermore, it

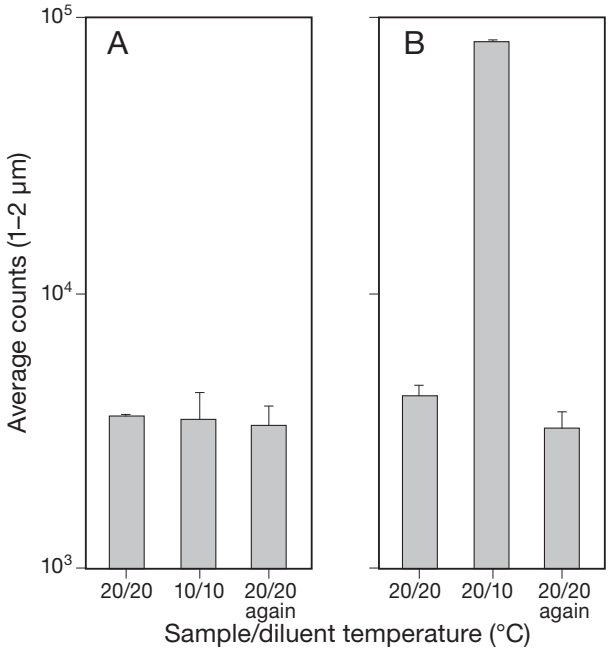

Fig. 1. Mean (±SE) cell counts in the 1–2 µm size range from (A) Series A and (B) Series B of diluted natural seawater samples. Each series consisted of 3 temperature combinations, each with 4 replicates. The first value for each combination is the temperature of the sample, the second is the temperature of the diluent (in °C). For Series A, all counts in the 1–2 µm range were measured when the sample and diluents were at the same temperature. For Series B, there was a 10°C difference between the sample temperature and the diluents during this middle trial, with only the sample cooled and re-warmed again. None of the counts in Series A are significantly different from each other ($\alpha = 0.05$), whereas the counts in the second trial in Series B were 2 orders of magnitude higher than the other trials

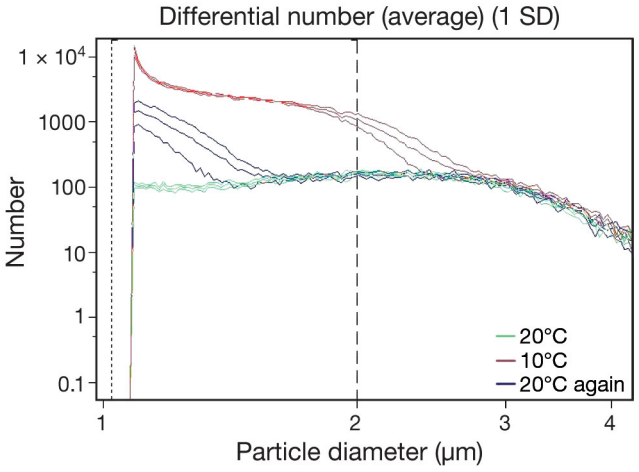

Fig. 2. *Synechococcus bacillaris*. Screen capture of results from the Coulter Counter Multisizer 3. Particle counts for 5 replicates of *S. bacillaris* (at 1:100 dilution from a log-phase growth culture) were measured at 20°C (green), 10°C (red), and 20°C again (blue) between 1 and 4 µm. For each spectrum, the middle line represents the mean of 5 replicates, and outer lines represent 1 SD. All samples were diluted in 0.22 µm filtered Instant Ocean, and run under the same instrumental conditions. The diluent supply was at room temperature throughout the experiment (20°C) and <1 h elapsed between measurements. At 10°C, cell counts in the 1–2 µm size range (dashed lines) increased by 2236% on average above initial 20°C values, but then decreased to 350% of the initial 20°C values upon re-warming back to 20°C, with deviations from initial counts only below 1.5 µm particle size

is not likely that cooling the cultures to only 10°C would cause significant physiological stress to our phytoplankton cells, as *Synechococcus* sp. can be abundant in 2°C coastal water (Shapiro & Haugen 1988), and is maintained at 15°C in our lab.

As noted by Baretta & Malschaert (1985), there are several ways counts can increase: cell breakage or lysis, large shifts in conductivity, and differences in conductivity within a sample. However, based on our results, we conclude that the lack of increased counts when the diluent and sample are maintained at the same temperature, and the return to initial 20°C counts when samples were re-warmed to room temperature, cannot be explained by these mechanisms. Instead, we propose the difference is due to stabilized microbubbles.

According to Henry's Law, solubility of gas within a liquid is partly a function of temperature; cooler liquids hold more dissolved gas and bubbles must form to allow dissolved gas to escape as the liquid warms (e.g. Wallace & Hobbs 2006). Such thermal degassing in warming liquids was noted by Taib & Sorrell (2008) as a limitation of the model LS 230 Coulter Counter, but they did not indicate the specific temperature contrasts that produce the effect. Johnson & Cooke (1981) demonstrated that stabilized microbubbles in NSW can form as a result of organic compounds and metal ion complexes forming a surface film. While their study did not associate these bubbles with liquids changing temperatures, the authors noted that the stabilization by films promotes preservation of microbubbles with diameters between 0.75 and 2.25 µm. Since natural coastal water can be rich in organic colloids, microbubbles formed as the cold organic-rich water comes into contact with warmer diluents could be stabilized by organic films derived from colloids (Guo et al. 1995). Furthermore, it has been found that bubbles >2 µm almost immediately float to the surface of a fluid, and those much smaller dissolve in a few seconds due to surface tension (Yount et al. 1984). Thus, smaller microbubbles are preferentially retained while bubbles >2.25 µm float out or collapse (Johnson & Cooke 1981), allowing for the increased counts in the <2.38 µm size range

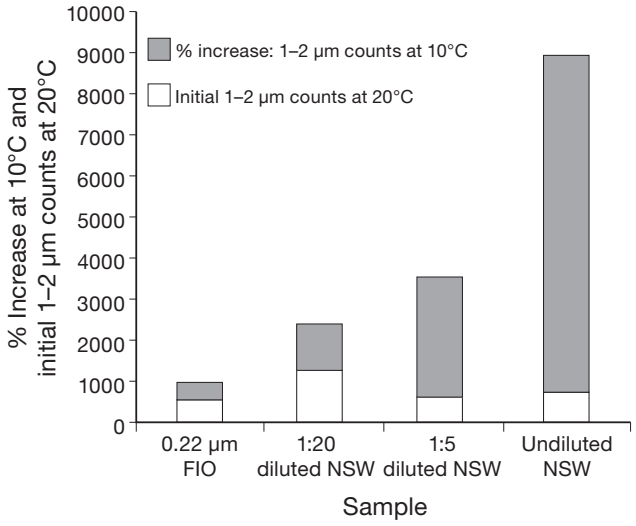

Fig. 3. Four replicates of natural seawater (NSW) were filtered with a 0.22 µm membrane filter and/or diluted to achieve comparable 1–2 µm counts as replicates of 0.22 µm filtered Instant Ocean (FIO). These samples and 4 replicates of 0.22 µm FIO were measured first at 20°C, then cooled to 10°C and re-measured. Despite similar 1–2 µm counts at 20°C, as the fraction of NSW increased, the percent amplification of 1–2 µm counts at 10°C increased ($r^2 = 0.84$, $p = 0.031$)

observed in the present study for the period of measurement (approximately 4 s) of the sample on the Multisizer 3 (Figs. 1 & 2).

The experiment diluting NSW strongly suggests that the effect is due to stabilizing organic compounds in the matrix of the seawater. Counts in the 1–2 µm range of 0.22 µm filtered NSW run at 10°C (with 20°C diluent) increased on average by 8183% (±1181% SE) from initial values at 20°C, while counts in the same size range of 0.22 µm FIO increased by only 381% (±110% SE) (Fig. 3). Counts of replicates of 0.2 µm filtered seawater diluted 1:5 with 0.22 µm FIO increased by 2903% (±174% SE) at 10°C compared to counts at 20°C. Despite similar initial counts in the 1–2 µm size range when the sample and diluent were maintained at 20°C, there was a linear increase in counts with the fraction of NSW relative to artificial seawater (FIO) when there was a 10°C difference between the sample and diluent ($r^2 = 0.84$, $p = 0.031$; Fig. 3). This suggests that amplification of counts in this small size range in cooled NSW may be a consequence of organic compounds stabilizing the surface of microbubbles in the <2.38 µm range and preventing their collapse.

CONCLUSIONS

Our results demonstrate that the formation of microbubbles can occur in natural and artificial seawater as cooler samples are warmed. These microbubbles could appear to be exponentially growing picophytoplankton or fluxes of small particles. To avoid this confounding effect, when counting small particles (<2.38 µm) in samples of a different temperature than the diluent, the samples should be allowed to equilibrate to ambient temperature, or the diluent should be cooled to the same temperature as the sample. If these actions are not taken, researchers may misinterpret their results as biological growth or increased small particle presence in marine systems. This overestimation would weaken the accuracy of models of future phytoplankton dynamics that currently indicate that picoplankton will become increasingly dominant in much of the ocean as it warms in response to climate change (Falkowski & Oliver 2007, Morán et al. 2009).

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