DIEL VARIATIONS IN OPTICAL PROPERTIES OF *MICROMONAS PUSILLA*, A PRASINOPHYTE

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ABSTRACT

A laboratory experiment was conducted on cultures of *Micromonas pusilla*, a marine prasinophyte, to investigate how cell growth and division affect the optical properties over the light:dark cycle. Measurements were made of cell size and concentration, attenuation and absorption coefficients, flow cytometric light scattering (in forward and side directions), chlorophyll and carbon content. Refractive index was calculated using the anomalous diffraction approximation. Cells were about 1.5 µm in diameter and exhibited phased division, with the major division burst occurring during the night. Typical diel variations were observed, with cells increasing in size and light scattering during the day as they photosynthesize and decreasing at night upon division. The cells were in ultradian growth, with more than one division per day, at a light level of 120 µmol photons m^{-2} s^{-1}. Since these cells are similar in size to small phytoplankton that are typically abundant in field samples, these results can be used in the interpretation of diel variations in light scattering in natural populations of phytoplankton.

INTRODUCTION

Diel variations in bulk optical properties such as beam attenuation (Siegel et al. 1989; Hamilton 1990; Cullen et al. 1992; Stramska and Dickey 1992; Gardner et al. 1993, 1995) and in single cell optical properties such as phytoplankton forward light scattering (Olson et al. 1990; DuRand 1995; DuRand and Olson 1996; Vaulot and Marie 1999) have been observed in numerous field studies in many of the world's oceans. A number of researchers have observed diel variations in individual cell light scattering during laboratory studies of various phytoplankton cultures (Stramski and Reynolds 1993; Stramski et al. 1995; DuRand and Olson 1998). The common pattern exhibited is minima at dawn and maxima near dusk. Researchers have related diel variations in beam attenuation to primary production (Siegel et al. 1989; Cullen et al. 1992; Walsh et al. 1995) and have calculated the contribution of different groups of organisms to beam attenuation in the ocean (DuRand 1995; DuRand and Olson 1996; Chung et al. 1998).

Light scattering is determined by a particle's size and refractive index and, to a lesser extent, shape and internal structures (Jerlov 1976). Contrasting data have been obtained on the relative contributions of variations in particle size and in refractive index to diel changes in optical cross sections. If diel changes in optical cross sections can be related to changes in cell size and carbon content, then phytoplankton productivity (Cullen et al. 1992) and growth rates (DuRand 1995; Binder et al. 1996) could be determined from measurements over the diel cycle in the field. Ackleson et al. (1993) reported carbon-independent changes in cell scattering in several phytoplankton cultures

that were attributed to cell swelling in response to high-light conditions. Stramski and Reynolds (1993) calculated that changes in refractive index were equal to or more important than changes in cell size in determining the attenuation cross section for the diatom *Thalassiosira pseudonana*. For this culture, the carbon-specific beam attenuation (c^*_c) changed by up to 30% over three diel cycles, but had no consistent diel pattern. In a laboratory study on a slowly growing culture of the prokaryotic picoplankton *Synechococcus*, Stramski et al. (1995) saw variations in the c^*_c of up to two-fold over the course of two days (though ~30% increase from dawn to dusk on each day). DuRand and Olson (1998) studied the chlorophyte *Nannochloris* over the diel cycle at a number of different light-limited growth rates and reported that the small changes in the calculated refractive index were not as important as cell size in determining diel variations in beam attenuation. In addition, the c^*_c exhibited an ~25% increase from dawn to dusk.

The present study on the small (~1.5 µm diameter) prasinophyte, *Micromonas pusilla*, was undertaken in order to study variations in optical and chemical properties over the diel cycle and to determine the relative importance of cell size and refractive index to changes in optical properties. *Micromonas* was chosen since it is a prasinophyte, which is common in the world's oceans, and is of a small size similar to that of eukaryotic ultraphytoplankton frequently observed using flow cytometry in field samples.

METHODS

Laboratory measurements - *Micromonas pusilla* (CCMP 489) was grown in f/2-Si medium in replicate batch cultures at 25°C. The filtered (0.22 µm) seawater and nutrient solutions were autoclaved separately and then added together after the seawater had cooled. In order to ensure particle-free medium, the solution was then 0.22 µm sterile-filtered. The cultures were grown in 10-liter carboys (containing 2 liters of culture at the start of sampling) in an incubator on a 12 h light:12 h dark cycle at PAR of 120 µmol photons m^{-2} s^{-1} (Cool-White fluorescent lights, measured with a Biospherical Instruments QSL-100 4π sensor). The carboys were bubbled with moisturized, filtered air. Samples were forced out by air pressure through a sterile sampling port. The cultures were diluted daily for 5 days prior to the day of sampling to maintain the cells in exponential growth.

Samples were withdrawn every 2 hours (starting at dawn, until the following dawn) and measurements made of cell concentration, forward and side light scattering, and chlorophyll fluorescence (flow cytometer), cell size (Coulter Multisizer), absorption (spectrophotometer), attenuation (ac-9), extracted chlorophyll content, and carbon content (CHN analyzer). Samples were processed in dim light during the nighttime sampling points.

A modified Epics V flow cytometer (Coulter Electronics Corp.) with a Cicero acquisition interface (Cytomation, Inc.) was used to measure forward light scattering (FLS, 3-19° at 488 nm), side angle light scattering (SSC, 54-126° at 488 nm) and chlorophyll fluorescence (660-700 nm) of the *Micromonas* cells in undiluted duplicate samples. Polystyrene microspheres of two sizes (0.66 µm and 2.14 µm, YG calibration-grade beads from Polysciences, Inc.) were added as internal standards and differentiated from cells by their orange fluorescence (555-595 nm). The samples were delivered with a peristaltic pump (Harvard Apparatus) and cell concentration was determined from pump flow rate and sample analysis time. Computer analysis of the listmode files was

done using a modified version of "CYTOWIN" software (originally written by D. Vaulot; http://www.sb-roscoff.fr/Phyto/cyto.html) to calculate mean values of the measured parameters normalized to reference beads (2.14 µm). Cell concentrations for three time points were determined from preserved samples, due to difficulties with the pump (live and preserved counts for several other time points were found to be comparable).

A Coulter Multisizer equipped with a 30-µm aperture was used to determine the size distribution of the *Micromonas* cells. The culture was diluted with filtered seawater (9- to 14-fold dilutions) in order to obtain coincidence rates of 6% or less. The size distributions of replicate samples were averaged and normalized to the cell concentration from flow cytometric analysis. These average 256-channel data of cell-diameter distributions were used to calculate the geometric projected area of the mean cell ($\overline{G}$), the diameter of the mean cell ($\overline{D}$), and the volume of the mean cell ($\overline{V}$) to include the effects of polydispersion in our calculations (equations from Stramski and Reynolds 1993; Reynolds et al. 1997).

Absorption was measured in a 1-cm cuvette using a Perkin-Elmer Lambda 18 UV/VIS spectrophotometer with a 60-mm integrating sphere. Syringe-filtered (0.22 µm) culture was used in the reference cuvette and as the blank. A dilution series was measured at the start of the experiment in order to ensure that, in subsequent measurements, multiple scattering effects were negligible.

An absorption and attenuation meter with a 25-cm pathlength (ac-9, WETLabs) was used to measure spectral attenuation at nine wavelengths (412, 440, 488, 510, 532, 555, 650, 676, and 715 nm). Two reservoirs were attached with tubing to the inlet and outlet of the ac-9, samples were gravity fed through the instrument, and data collection was closely monitored to ensure the absence of bubbles. A dilution series was measured at the start of the experiment to ascertain that we were operating within the linear range of the instrument. Phytoplankton cultures were diluted 9- to 21-fold with filtered seawater before measurement. Filtered seawater was run between consecutive samples and subtracted as a blank. Temperature and salinity corrections were applied to the attenuation values (Pegau et al. 1997).

Triplicate samples of 1-ml culture were filtered onto GF/F filters and extracted in 90% acetone. Samples were analyzed on a Turner 10-AU fluorometer (with optical kit 10-040R) using the non-acidification technique (Welschmeyer 1994).

Duplicate samples of 25-ml culture were filtered onto pre-combusted GF/F filters, frozen, and later dried overnight at 60°C and analyzed on a Perkin-Elmer 2400 CHN analyzer with acetanilide as the standard.

Calculations of optical properties - The anomalous diffraction approximation (Van de Hulst 1957) was used to calculate efficiency factors for absorption and attenuation and then the imaginary part of the refractive index (n') and refractive index (n) were calculated through iteration (following equations in Morel and Bricaud 1986; Bricaud and Morel 1986). The measured values used for these calculations are the absorption and attenuation coefficients, cell concentrations, and cell diameter distributions. Calculations were made at both 488 and 650 nm. Assumptions of the theory include that the particles are homogenous and spherical, with a refractive index close to that of the surrounding medium, which is reasonable for these phytoplankton cells.

RESULTS AND DISCUSSION

Micromonas pusilla cell division was phased to the light:dark cycle with most of the division occurring during the first eight hours of darkness (Fig. 1A). Nighttime division is common among eukaryotic phytoplankton in the ocean (Chisholm 1981). The cultures in this experiment were in ultradian growth, with a growth rate over the 24-hr experiment of 0.9 d^{-1} (1.3 divisions per day) at a light level of 120 µmol photons m^{-2} s^{-1}. A portion of this division occurred during the light period, but the majority of it took place during the dark period (approximately one doubling per day from dusk to dawn).

The diameter of the mean cell ($\overline{D}$) and the volume of the mean cell ($\overline{V}$) both increased during the day as the cells were photosynthesizing and growing in size, and then decreased at night upon cell division (Fig. 1B,C). The flow cytometric analysis showed similar patterns in forward light scattering (FLS, expressed relative to 2.14 µm beads), side scattering (SSC), and also in chlorophyll fluorescence (Fig. 2). Preliminary results from the CHN analysis indicate that the carbon per cell was also minimum at dawn and maximum at dusk (data not shown).

The cross sections for absorption and attenuation obtained by normalizing the coefficients to the cell concentration also increased during the day and decreased during the night (Fig. 3A,C). The scattering cross section (Fig. 3B) was obtained by subtraction and was found to be the major part of the attenuation cross section (78-84%).

The calculated imaginary part of the refractive index (n', at 488 nm) varied from 0.007 to 0.011 without any discernable diel pattern (Fig. 4A). The real part of the refractive index (n, at 488 nm) varied from 1.05 to 1.07 and also exhibited no diel pattern (Fig. 4B). Most of the variability in both n' and n occurred between the first sampling point ("dawn") and the next sampling point (two hours later).

There was a strong relationship between cell volume and FLS with $r^2 = 0.91$ (n=26) and between FLS and the attenuation cross section with $r^2 = 0.94$ (n=26) (Fig. 5). Carbon per cell was strongly correlated with cell volume, with FLS, and with the attenuation cross section, indicating that increases in volume (and FLS and σ_c) are caused by real increases in carbon content of the cells as they photosynthesize and fix carbon.

Results from this diel sampling experiment on *Micromonas* indicate that the cells increased in cell size and carbon content during the light period and then decreased in size with cell division during the dark period. Flow cytometric forward and side light scattering followed the same diel pattern, as did cross sections for attenuation, scattering and absorption. The refractive index, calculated using the anomalous diffraction approximation, did not show any significant trend with the light:dark cycle.

Since the single-cell measurements of forward light scattering were strongly correlated with independent measurements of both cell volume and attenuation cross section, this data set could help provide calibrations for using flow cytometric measurements of similar phytoplankton made at sea to estimate cell size and growth rates and contributions to bulk optical properties such as beam attenuation.

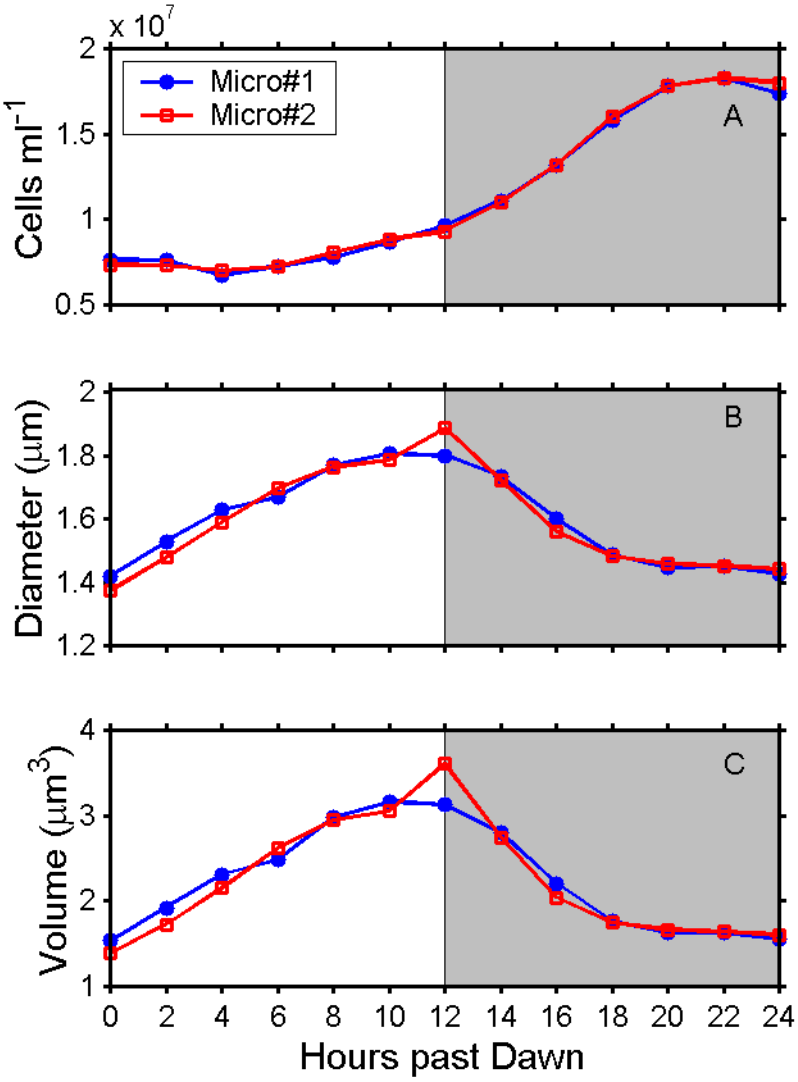

Figure 1. Time series of A) cell concentration, B) cell diameter ($\overline{D}$), and C) cell volume ($\overline{V}$) for replicate carboys (#1 and #2) of Micromonas cultures. The shaded area denotes when the lights were off in the incubator (12-24 hours past dawn).

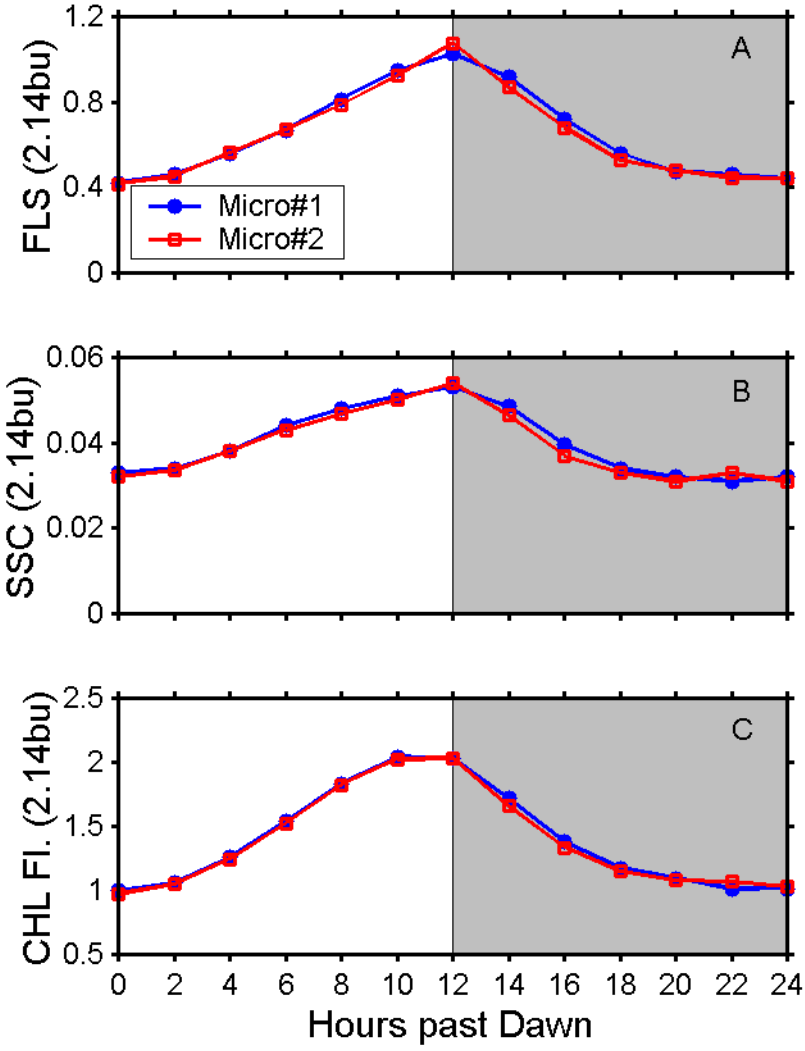

Figure 2. Time series of flow cytometric data expressed relative to 2.14 µm beads. A) forward light scattering (FLS), B) side scattering (SSC), and C) chlorophyll fluorescence for replicate carboys (#1 and #2) of Micromonas cultures. The shaded area denotes the dark period.

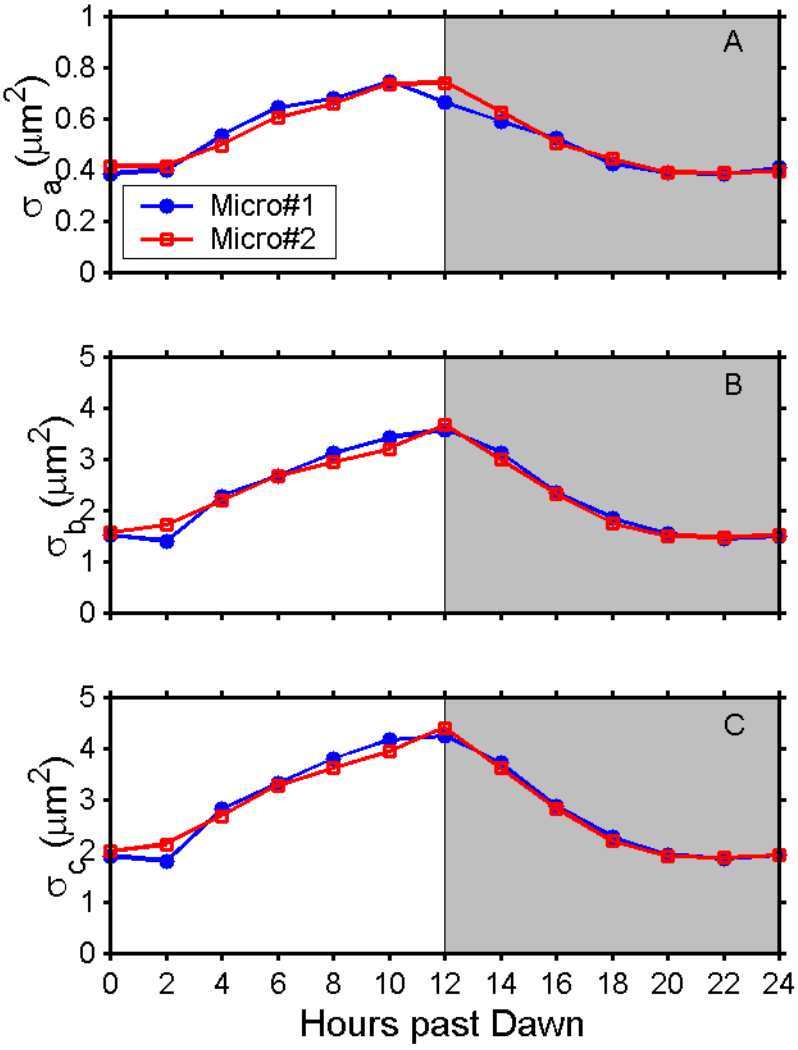

Figure 3. Time series of optical cross sections (in μm^2 at 488 nm) for A) absorption (σ_a), B) scattering (σ_b, obtained from σ_c - σ_a), and C) attenuation (σ_c) for replicate carboys (#1 and #2) of Micromonas cultures. Note the different y-axis scaling in panel A. The shaded area denotes the dark period.

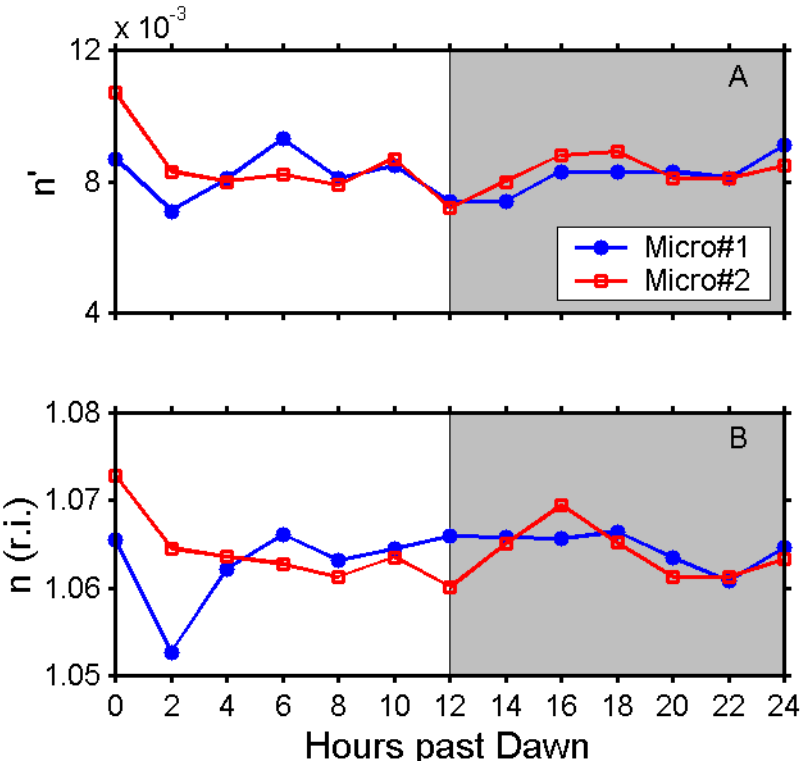

Figure 4. Time series of A) the imaginary part of the refractive index (n', at 488 nm) and B) the refractive index (n, at 488 nm) for replicate carboys (#1 and #2) of Micromonas cultures. Calculations were made using the anomalous diffraction approximation.

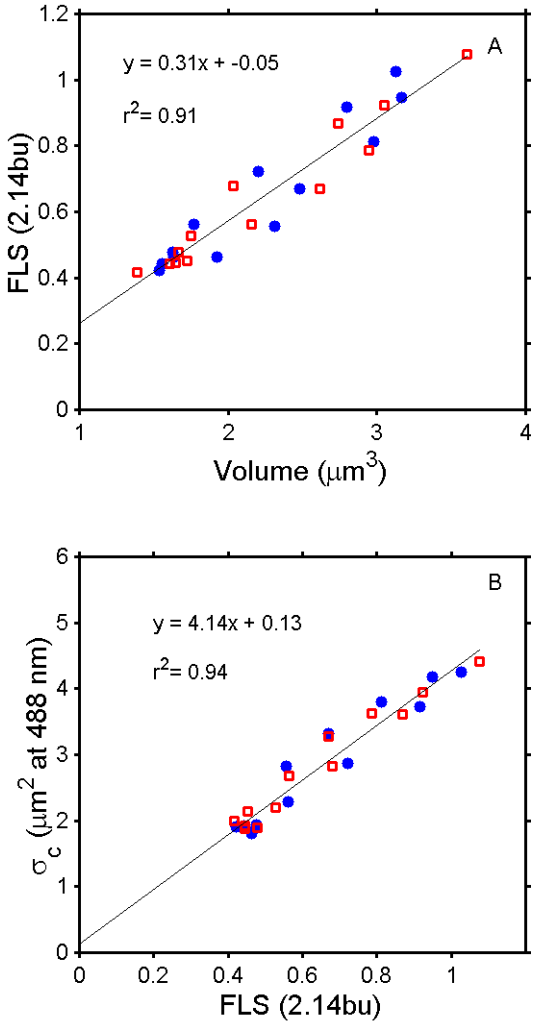

Figure 5. A) Relationship between forward light scattering (3-19°, relative to 2.14 µm beads) and cell volume (in µm^3) with linear regression. B) Relationship between attenuation cross section (σ_c in µm^2 at 488 nm) and forward light scattering (3-19°, relative to 2.14 µm beads) with linear regression. Micromonas carboy #1 data are shown in solid blue circles, carboy #2 data are in open red squares.

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