Direct single-cell biomass estimates for marine bacteria via Archimedes’ principle

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Running title: Single-cell biomass estimation

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Abstract

Microbes are an essential component of marine food webs and biogeochemical cycles, and therefore precise estimates of their biomass are of significant value. Here, we measured single-cell biomass distributions of isolates from several numerically abundant marine bacterial groups, including *Pelagibacter* (SAR11), *Prochlorococcus*, and *Vibrio* using a microfluidic mass sensor known as a suspended microchannel resonator (SMR). We show that the SMR can provide biomass (dry mass) measurements for cells spanning more than two orders of magnitude, and that these estimates are consistent with other independent measures. We find that *Pelagibacterales* strain HTCC1062 has a median biomass of 11.9±0.7 fg cell\(^{-1}\), which is five- to twelve-fold smaller than the median *Prochlorococcus* cell’s biomass (depending upon strain), and nearly 100-fold lower than that of rapidly growing *V. splendidus* strain 13B01. Knowing the biomass contributions from various taxonomic groups will provide more precise estimates of total marine biomass, aiding models of nutrient flux in the ocean.
Introduction

Per-cell microbial biomass estimates are extremely important in parameterizing ecological and biogeochemical models (Ducklow, 2000). Beyond the average, the full distribution of single-cell biomass may also be important in biophysical models. However, single-cell biomass is non-trivial to determine. Established techniques include CHN analyzers (Lee and Fuhrman, 1987) and high-temperature catalytic oxidation (Fukuda et al., 1998), which when combined with cell counts can be used to estimate average biomass and elemental mass per cell. Alternatively, transmission electron microscopy, x-ray microanalysis, and particle volume sensors based on the Coulter principle (also known as resistive pulse sensing) provide single cell mass or volume distributions (e.g., Fagerbakke et al., 1996; Kogure and Koike, 1987; Loferer-Krößbacher et al., 1998). However, particle volume sensors are generally not sensitive enough to resolve the smallest marine bacteria, and TEM-based analyses are difficult to scale up since they require significant labor, technical skill, and image processing.

Here, we demonstrate the use of a micromechanical mass sensor to measure the single-cell biomass (dry mass) distributions of isolates from several ubiquitous marine bacterial groups including Pelagibacter (SAR11), Prochlorococcus, and Vibrio. The SAR11 clade is estimated to have a global abundance of $2.4 \times 10^{28}$ cells, and is the most abundant marine bacterial group (Morris et al., 2002). Prochlorococcus is the most abundant primary producer on Earth with a global estimate of $2.9 \times 10^{27}$ cells (Flombaum et al., 2013) and supports a significant fraction of the secondary production that occurs in warm oligotrophic surface waters. Unlike Pelagibacter and Prochlorococcus, which are abundant open-ocean organisms (Flombaum et al., 2013; Morris et al., 2002; Partensky et al., 1999), Vibrio is commonly found in more productive waters at concentrations $\sim 10^3$ cells mL$^{-1}$ (Takemura et al., 2014); however, massive, short-lived blooms have recently been documented, during which vibrios can represent dominant community members (up to 50% of total bacteria) (Gilbert et al., 2012; Westrich et al., 2016).
To measure single-cell biomass, we used suspended microchannel resonators (SMRs) - microcantilever-based microfluidic mass sensors that directly measure single-cell buoyant mass (Burg et al., 2007). The SMR consists of a hollow vibrating microcantilever with an internal microfluidic channel, which changes its resonant frequency proportionally to a cell’s buoyant mass whenever a cell flows through the interior of the cantilever. A cell’s buoyant mass is its total mass minus the mass of the fluid it displaces. To obtain dry mass (biomass), we combine information from paired buoyant mass measurements performed in H₂O and D₂O (Feijó Delgado et al., 2013). In pure H₂O, a cell’s buoyant mass is only the buoyant mass of its dry material, as its intracellular water is neutrally buoyant. Similarly, in heavy water (D₂O) – which permeates the cell and replaces internal H₂O – a cell’s buoyant mass is also only the buoyant mass of its dry material. We exploit this property to obtain the density of a cell’s dry material (termed its dry density), with which we can convert from buoyant mass in H₂O or D₂O to biomass (Feijó Delgado et al., 2013), as shown in Figure 1A. We fixed cells so they would not lyse under hypoosmotic conditions, resuspended them in H₂O or D₂O, and then measured their buoyant mass distributions. We then use these distributions to calculate the single cell biomass distributions and uncertainty in their associated statistics (supplementary methods).

**Results and discussion**

Previous work on natural bacterial assemblages has found nearly three orders of magnitude variation in single cell biomass, from three femtograms to over a picogram (Loferer-Krößbacher et al., 1998). In accordance with this natural variation, we find that median biomass varies nearly 100-fold between cultivated isolates from abundant marine bacterial clades. *Pelagibacter* median single cell biomass was between 12 and 16 fg, *Prochlorococcus* between 60 and 158 fg, and *V. splendidus*, depending on the growth stage, between 150 and 1000 fg (Figure 1B, Table 1). These values are consistent both with our measurements of buoyant mass in seawater-based media (Figure S1) and with
literature values, summarized below. Upon initial cultivation, *Pelagibacterales* strain HTCC1062 was reported to be extremely small, with an estimated cell volume of ca. 0.01 µm³ determined by TEM (Rappé et al., 2002). The carbon content of HTCC1062 was later estimated at 5.8 fg C per cell (Tripp et al., 2008), which corresponds to 11.6 fg of total biomass if carbon accounts for half the cell's biomass. Our direct estimates of single-cell biomass for HTCC1062 and HTCC7211 are consistent with these previous reports and support the notion that *Pelagibacterales* are among the smallest known free-living cells. Previous estimates of *Prochlorococcus* biomass range from 15 to 94 fg C cell⁻¹ (or 30-188 fg total biomass, assuming 50% carbon content), and were derived from strains belonging to the HLI clade (Bertilsson et al., 2003; Buitenhuis et al., 2012), the same as strain MED4 used here. Here we find median dry mass for *Prochlorococcus* to be between 60 and 158 fg, with higher values corresponding to the first direct biomass measurements of low light-adapted *Prochlorococcus* (NATL2A and MIT9313), which we find can be >2-fold higher than their high light-adapted relatives. We also note that across our *Prochlorococcus* and *Pelagibacter* strains, biomass increases monotonically with genome size (Figure 1C).

To our knowledge, the dry mass of *Vibrio splendidus* has not been previously measured; however x-ray microanalysis of *Vibrio natriegens* yielded a geometric mean dry mass of 850 fg for exponential-phase cells and 145 fg for stationary-phase cells (Fagerbakke et al., 1996). Such drastic differences between exponential and stationary phase cells – exceeding 5-fold mass changes – have also been observed in *E. coli* (Feijó Delgado et al., 2013; Loferer-Krößbacher et al., 1998), and are correlated with a substantial reduction in RNA:protein ratio.

Our measurement also provides information on within-strain size variation. Strikingly, we found that the coefficient of variation (estimated using a robust metric – see supplementary methods) was highly consistent across strains, ranging from 26-30%. For unsynchronized cells, deterministically growing either linearly or exponentially from mass $m_0$ to $2m_0$ and then dividing symmetrically, one
would expect a robust CV of ~25%. While we expect our *Pelagibacter* and *Vibrio* populations to be unsynchronized, the *Prochlorococcus* strains were grown under diel light conditions and thus were fixed toward the end of the day, just before division begins, so likely at their maximal size. This suggests that unsynchronized *Prochlorococcus* would likely have a broader size distribution than *Pelagibacter* or *Vibrio*. Estimates of cell-to-cell mass variation may be useful in constraining biophysical models of marine microbial behavior, and could ultimately inform how uniquely a mass identifies a microbe or its growth state.

Our results show that SMR can provide single cell biomass estimates spanning nearly two orders of magnitude among marine bacteria, a variation that needs to be taken into account when considering the importance of different taxonomic groups in the global carbon cycle. Moreover, *Pelagibacter* and *Prochlorococcus* strains also demonstrate considerable biomass variation within taxonomic groups that may reflect the ecological constraints different ecotypes or populations live under. We propose that SMR micromechanical mass sensors are an efficient means to determine biomass under different ecological conditions to further refine estimates of global microbial biomass.

**Acknowledgments**

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References:


Table 1. Summary statistics for the biomass distributions shown in Fig 1B. Robust CV: robust coefficient of variation, calculated as \(0.741 \times \text{interquartile range/median.}\)

<table>
<thead>
<tr>
<th>Strain</th>
<th>N</th>
<th>Median (fg)</th>
<th>10%</th>
<th>90%</th>
<th>Robust CV (%)</th>
<th>Dry density (g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pelagibacter</strong></td>
<td></td>
<td></td>
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<tr>
<td>HTCC1062</td>
<td>1325</td>
<td>11.9±0.7</td>
<td>9</td>
<td>17</td>
<td>30.1±1</td>
<td>1.48±0.04</td>
</tr>
<tr>
<td>HTCC7211</td>
<td>1989</td>
<td>16±0.8</td>
<td>13</td>
<td>23</td>
<td>25.7±1</td>
<td>1.52±0.03</td>
</tr>
<tr>
<td>MIT9301 (HLII)</td>
<td>818</td>
<td>60±3</td>
<td>44</td>
<td>87</td>
<td>29±1</td>
<td>1.35±0.02</td>
</tr>
<tr>
<td>MED4 (HLI)</td>
<td>1177</td>
<td>66±4</td>
<td>49</td>
<td>94</td>
<td>30±1</td>
<td>1.39±0.02</td>
</tr>
<tr>
<td>NATL2A (LLI)</td>
<td>1154</td>
<td>91±5</td>
<td>69</td>
<td>127</td>
<td>26.3±0.9</td>
<td>1.42±0.03</td>
</tr>
<tr>
<td>MIT9313 (LLIV)</td>
<td>1936</td>
<td>158±6</td>
<td>120</td>
<td>216</td>
<td>26.9±0.8</td>
<td>1.43±0.02</td>
</tr>
<tr>
<td><strong>Prochlorococcus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Vibrio</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Stationary 13B01</td>
<td>1875</td>
<td>150±8</td>
<td>107</td>
<td>200</td>
<td>26±0.7</td>
<td>1.51±0.03</td>
</tr>
<tr>
<td>Exponential 13B01</td>
<td>817</td>
<td>1000±100</td>
<td>750</td>
<td>1530</td>
<td>29±1</td>
<td>1.58±0.08</td>
</tr>
</tbody>
</table>

Figure 1. Measuring single-cell biomass (dry mass) of marine microbial isolates via Archimedes’ principle. **(a)** Paired measurements of a population of cells in H\(_2\)O (\(\rho_{\text{fluid}} = 1.0\) g/mL) and D\(_2\)O (\(\rho_{\text{fluid}} = 1.1\) g/mL).
g/mL yields the dry density of the population, enabling conversion of buoyant mass distributions to dry mass distributions. (b) Biomass distributions for various cell types. (c) Log-log plot of genome size vs median single-cell biomass. Colors are as in (b)
A

Buoyant mass, $m_b$

$m_b = m(1 - \rho_{\text{fluid}}/\rho_{\text{dry}})$

Fluid density, $\rho_{\text{fluid}}$ (g/mL, not to scale)

B

SAR11 HTCC1062

Prochlorococcus MIT9301

Prochlorococcus MED4

Prochlorococcus NATL2A

Prochlorococcus MIT9313

Vibrio splendidus 13B01 stationary phase

Vibrio splendidus 13B01 exponential phase

C

Median dry mass (fg)

Genome size (Mbp)

Biomass (fg)
Supplementary methods

Suspended microchannel resonator

A 120 micron long suspended microchannel resonator was used, operated in the second mode at 2.1 MHz. The cross-section of the device’s interior fluidic channel was 3x5 µm². The device was calibrated with 1.1 µm polystyrene particles and NaCl density standards prior to use.

Data analysis and calculation of dry mass

As detailed in Feijó Delgado et al., 2013, a cell’s buoyant mass in H₂O and D₂O are given as follows:

\[ m_{b,H_2O} = m_{dry} \left( 1 - \frac{\rho_{H_2O}}{\rho_{dry}} \right) \]  
\[ m_{b,D_2O} = m_{dry} \left( 1 - \frac{\rho_{D_2O}}{\rho_{dry}} \right) \]

Where \( m_{b,H_2O} \) is a cell’s buoyant mass in H₂O, \( m_{dry} \) is the cell’s dry mass, and \( \rho_{dry} \) is the cell’s dry density (the density of only its biomass). Measurements of both \( m_{b,H_2O} \) and \( m_{b,D_2O} \) are sufficient to solve for \( m_{dry} \) and \( \rho_{dry} \) as follows:

\[ m_{dry} = \frac{\rho_{D_2O} m_{b,H_2O} - \rho_{H_2O} m_{b,D_2O}}{\rho_{D_2O} - \rho_{H_2O}} \]
\[ \rho_{dry} = \frac{\rho_{D_2O} m_{b,H_2O} - \rho_{H_2O} m_{b,D_2O}}{m_{b,H_2O} - m_{b,D_2O}} \]

We take the median buoyant mass of a strain in H₂O or D₂O to be \( m_{b,H_2O} \) or \( m_{b,D_2O} \), respectively, and thus calculate median dry mass and dry density. We assess our uncertainty in both dry density and median biomass by bootstrapping this process 1000 times (resampling the H₂O and D₂O buoyant mass distributions and recalculating our statistics each time).
To obtain the distributions shown in Figure 1B, we calculated a strain’s dry density based on its median dry masses, and then converted each single-cell buoyant mass measurement to dry mass using equations (1) and (2).

The robust coefficient of variation was calculated using the ratio of two robust statistics, the interquartile range over the median. We then rescale this statistic by 0.741, such that for the normal distribution, this estimate is consistent with the non-robust estimator of the sample standard deviation over the sample mean.

**Cell culture and fixation:**

*Pelagibacterales* strains HTCC1062 and HTCC7211 were obtained from Stephen Giovannoni. HTCC1062 cells were grown in AMS1 (Carini et al., 2013) with the following additions/modifications: 1 mM NH₄Cl, 10 µM KH₂PO₄, 1 µM FeCl₃, 25 µM Glycine, 25 µM methionine, 100 µM pyruvate and the following mixed vitamins (1 µM pantothenate, 1 nM biotin, 1 nM PQQ, 1 nM HMP and 1 nM B12). The culture was fixed by adding formaldehyde (0.37% final concentration) and immediately storing it at 4°C until processing. HTCC7211 was grown in AMS1 supplemented with 50µM pyruvate, 50µM glycine and 10 µM methionine. Cells were fixed with glutaraldehyde (0.125% final concentration), incubated in the dark for 10 min, and stored at 4°C until processing.

*Prochlorococcus* strains were grown in natural Sargasso seawater-based Pro99 medium (Moore et al., 2007) at 24°C under a 13-h/11-h light (10 µmol quanta m⁻² s⁻¹)/dark cycle. Cultures were fixed the same way as HTCC7211. All *Pelagibacterales* and *Prochlorococcus* strains were between 1-4 x 10⁷ mL⁻¹ at the time of fixation.

*Vibrio splendidus* 13B01 was grown in 1mL 2216 Marine Broth (Difco, BD) for ~18 h at room temperature under continuous shaking. Stationary phase cells were harvested from the 18 h culture, while exponential phase cells were obtained after diluting the stationary phase cells 1000-fold and
allowing 5 h of growth (OD$_{600}$ of approximately 0.1). Fixation was identical to HTCC7211, but with 1.25% glutaraldehyde.
Supplementary Figure legend:

**Figure S1.** Buoyant mass distributions in media (natural or artificial seawater-based media for SAR11 and *Prochlorococcus*, Marine Broth 2216 for *Vibrio*).