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SHORT COMMUNICATION

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

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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 per cell, 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.

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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 (for example, Kogure and Koike, 1987; Fagerbakke *et al.*, 1996; 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 2.4×10^{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 2.9×10^{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 (Partensky *et al.*, 1999; Morris *et al.*, 2002; Flombaum *et al.*, 2013), *Vibrio* is commonly found in more productive waters at concentrations ~10^3 cells per ml (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

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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 H_2O and D_2O (Feijó Delgado *et al.*, 2013). In pure H_2O, 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 (D_2O)—which permeates the cell and replaces internal H_2O—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 H_2O or D_2O 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 H_2O or D_2O 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 the 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 (Supplementary Figure S1) and with literature values, which is summarized below. Upon initial cultivation, *Pelagibacterales* strain HTCC1062 was reported to be extremely small, with an estimated cell volume of ca. 0.01 μm^3 determined by TEM (Rappe *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-

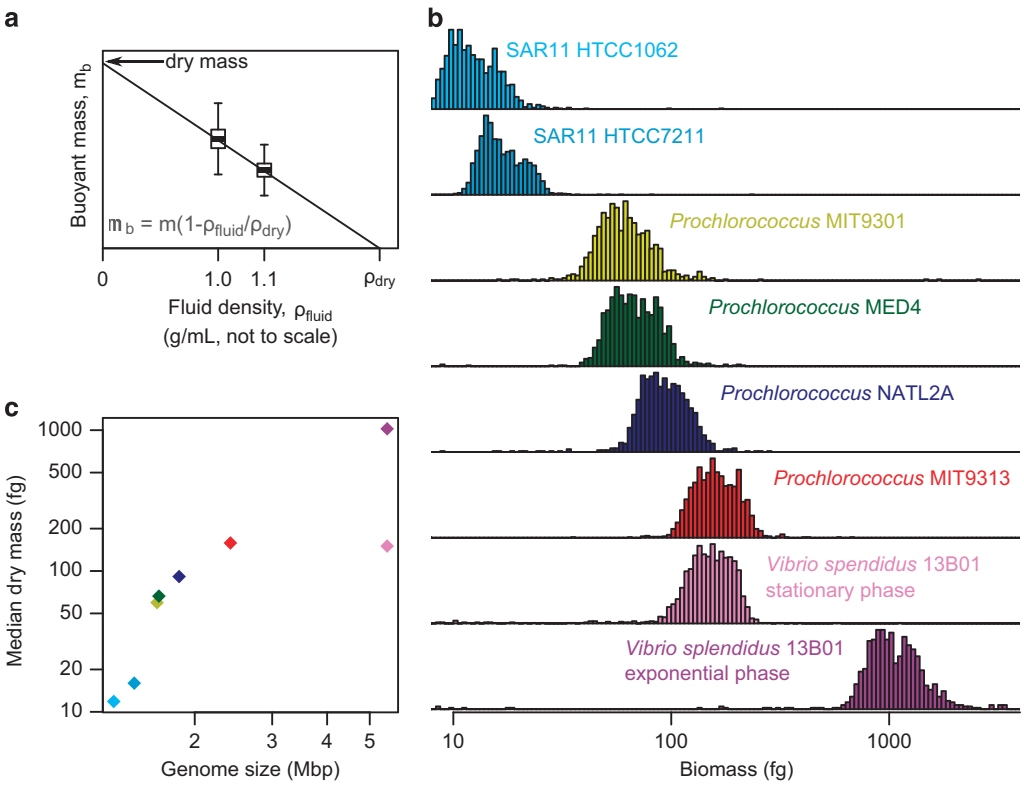

Figure 1 Measuring single-cell biomass (dry mass) of marine microbial isolates via Archimedes' principle. (**a**) Paired measurements of a population of cells in H_2O ($\rho_{fluid} = 1.0$ g ml^{-1}) and D_2O ($\rho_{fluid} = 1.1$ g ml^{-1}) yields the dry density of the population, enabling conversion 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**).

Table 1 Summary statistics for the biomass distributions shown in Figure 1b

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

Abbreviation: Robust CV, robust coefficient of variation.
Robust CV is calculated as 0.741 × interquartile range/median.

living cells. Previous estimates of *Prochlorococcus* biomass range from 15 to 94 fg C per cell (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 to 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 unsychronized, 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 that 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.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)

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 μm^2. 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 H_2O and D_2O are given as follows:

$$m_{b,H_2O} = m_{dry}\left(1 - \frac{\rho_{H_2O}}{\rho_{dry}}\right) \quad (1)$$

$$m_{b,D_2O} = m_{dry}\left(1 - \frac{\rho_{D_2O}}{\rho_{dry}}\right) \quad (2)$$

Where m_{b,H_2O} is a cell's buoyant mass in H_2O, m_{dry} is the cell's dry mass, and ρ_{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 ρ_{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 H_2O or D_2O 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 H_2O and D_2O 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 NH_4Cl, 10 μM KH2PO4, 1 μM $FeCl_3$, 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 50uM pyruvate, 50uM 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 *Prochloroccoccus* strains were between 1-4 x 10^7 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.

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Supplementary Figure:

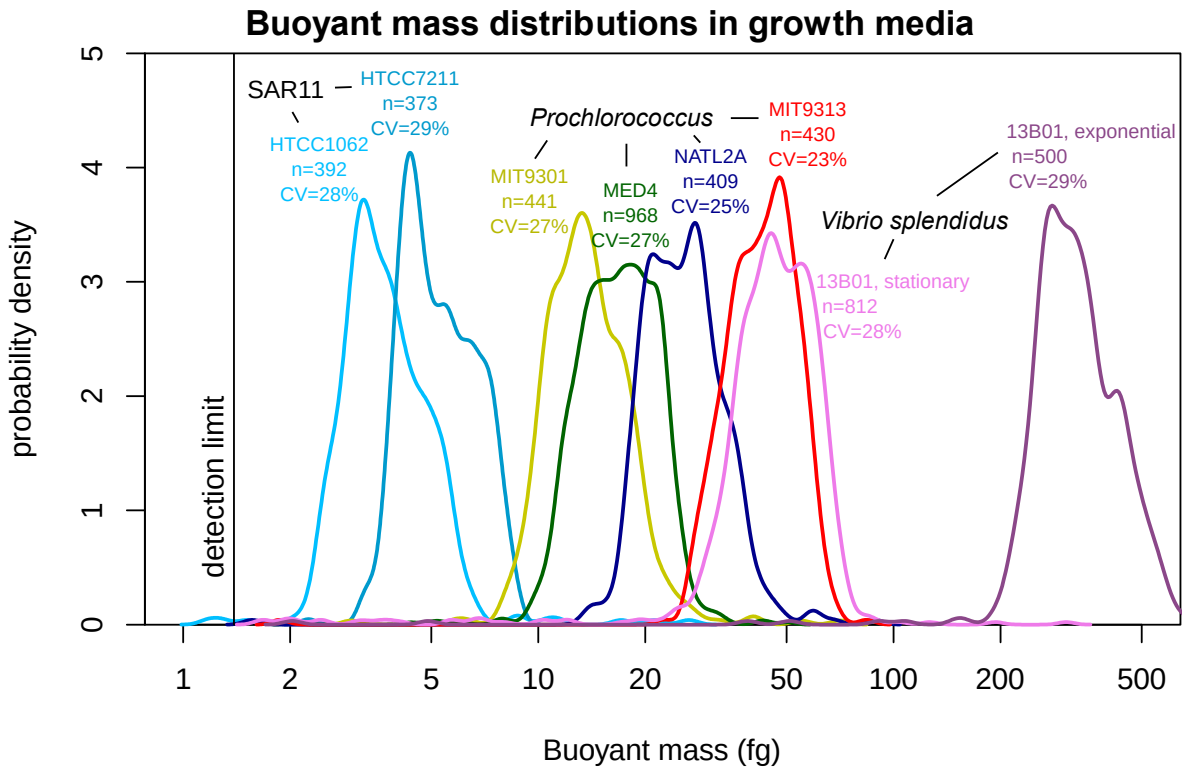

Figure S1. Buoyant mass distributions in media (natural or artificial seawater-based media for SAR11 and *Prochlorococcus*, Marine Broth 2216 for *Vibrio*).