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Bacterial Biovolume and Biomass Estimations

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The biomass of bacterial populations in aquatic ecosystems is often estimated by measuring bacterial biovolume and converting this into biomass in terms of carbon. A reliable conversion factor relating the measured bacterial biovolume to bacterial carbon content is essential for this approach. Based on direct measurements of bacterial cell carbon content, cell number, and biovolume, I have derived an average conversion factor of 5.6×10^{-13} g of C μm^{-3}. This conversion factor is 3.4 to 6.6 times higher than most theoretically derived factors currently in use. Both bacterial biomass and bacterial production in aquatic ecosystems may thus have been seriously underestimated.

The biomass of the bacterial population has been recognized as an important parameter when studying microbial ecology. Several approaches have been used to determine the bacterial biomass in natural aquatic ecosystems (for a review see reference 37). One approach that has received considerable attention is to measure the bacterial cell biovolume (volume per cell) and to convert this to bacterial cell biomass (as carbon per cell) by using a conversion factor (3, 6, 9, 26, 27, 29).

Several methods have been used in estimations of bacterial biovolume. These include epifluorescence microscopy (1, 2, 6, 13, 17, 20, 38), scanning electron microscopy (SEM) and transmission electron microscopy (3, 8, 9, 19, 43, 44), and electronic particle analyzers (23).

The bacterial population in any natural water sample will usually not constitute a discrete size class and overlaps in size distribution with populations of other small organisms, organic debris, inorganic particles, etc. A reliable size distribution of the bacterial population thus cannot be obtained by an electronic particle analyzer, unless the background number of nonbacterial particles is negligible. This condition is seldom satisfied, but it may occasionally be obtained during dense bacterial blooms in natural systems and in laboratory cultures.

Bacterial cells can easily be distinguished from other particles by the use of many microscopic techniques. Acridine orange staining combined with epifluorescence microscopy (AODC technique) is widely used for direct counting of bacteria in samples from natural environments. This technique has also been used for size measurements of bacteria (1, 2, 6, 13, 17, 20, 38). Owing to the limited optical resolution by light microscopy, the size and volume of very small cells may be difficult to measure (44).

Electron microscopy offers a much higher resolution, and the size of bacteria can accordingly be measured very precisely. However, methodological artifacts, such as shrinking and distortion of the cells, have been found to cause unpredictable errors in size estimations. (8, 23, 43). One disadvantage inherent in all microscopical size measurements is that small errors in measured length and width of the cells results in large errors when their volume is computed.

The use of conversion factors for estimating bacterial biomass from biovolume measurements is commonly based on the assumption that there is a constant carbon-to-biovolume ratio. Cell quotas of major cell constituents, including water, are, however, found to vary both with bacterial species and with growth condition (4, 22, 38). There is accordingly no valid reason to assume that the mass of carbon per unit of cell volume should be constant for bacteria. Several authors have calculated factors for the conversion of biovolume into biomass. These calculations are normally based on averaged values of published data for density and carbon-to-wet-weight ratio of bacteria or density, dry-weight-to-wet-weight ratio, and carbon-to-dry-weight ratio of bacteria (2, 3, 6, 13, 17, 39). Factors ranging from 0.847×10^{-13} to 1.65×10^{-13} g of C μm^{-3} have been published (3, 13). As far as I know, these conversion factors have never been experimentally verified.

In this paper I present experimentally derived factors for the conversion of bacterial biovolume into biomass. These conversion factors are based on direct measurements of bacterial cell carbon content, cell number, and biovolume. The validity of the basic assumption of a constant carbon-to-biovolume ratio was evaluted for pure and mixed cultures of bacteria grown under carbon, nitrogen, or phosphorus limitation. I have in this study also compared four different methods for biovolume determination.

MATERIALS AND METHODS

Media. Aged seawater was diluted to 70% with distilled water and filtered twice through filters (0.2-μm pore size; Sartorius) to remove any particles before autoclaving. My basal seawater medium contained glucose, equivalent to 5 mM carbon; $NaNO_3$, 0.3 mM; and KH_2PO_4, 20 μM. By increasing the concentration of two of the nutrients 10-fold and leaving the third nutrient unchanged, I obtained three different media designed to give carbon-, nitrogen-, or phosphorus-limited cells. Trace amounts of vitamins were added to all media (5).

Inocula and growth conditions. One series of the three different media was inoculated with *Pseudomonas putida* NCMB 1960. Another series was inoculated with a mixed population of bacteria collected from brackish water in an local estuary (Puddefjorden; Bergen). A water sample from this estuary was filtered twice through a filter (1-μm pore size; UniPore) to remove larger organisms and particles (algae, nanozooplankton, detritus) and was used as inoculum. The six cultures were incubated at 23°C on a rotary shaker and harvested in the late logarithmic phase of growth. To confirm the identity of the limited nutrient at the end of the incubation, I analyzed the culture filtrates for remaining glucose (Glox; Kabi Diagnostica, Sweden) and phosphate (for method see below). The amount of nitrogen remaining in

the medium was estimated as the difference between the amount of nitrogen initially added to the media and the nitrogen content of the cells (for method see below). For all six cultures I found that the nutrient intended to be limiting always was undetectable or very low in concentration compared with the concentration of the two other main nutrients.

Determination of cell carbon, nitrogen, and phosphorus. An amount of 10 to 40 ml of culture was filtered onto precombusted glass fiber filters (Whatman GF/F). The filters were dried and analyzed for carbon and nitrogen in a CHN analyzer (Carlo Erba Instrumentazione model 1106). Three to five replicate samples were analyzed for each culture. The coefficient of variation (CV) averaged 2.4% (range, 0.7 to 3.9%). Bacteria passing the glass fiber filter were counted as described below. Of the total number of bacteria in the samples, 0.01 to 1.3% (average, 0.3%) were found to pass the filters. Some of the culture filtrates were refiltered to determine the background caused by unused glucose and nitrate remaining in the medium. Carbon background caused by glucose was found to be 0.6 to 12.3% (average, 5.3%) of total carbon in the samples, and nitrogen background caused by nitrate was found to be 0.4 to 8.2% (average, 3.0%) of total nitrogen in the samples. Both these sources of error in the estimates of cell carbon and nitrogen were corrected for. Phosphorus in bacteria was estimated as the difference between total phosphorus and soluble reactive phosphate in the cultures after incubation. Total phosphorus and soluble reactive phosphorus were determined according to the method of Koroleff (18). Two to five replicate samples were analyzed for each total phosphorus and soluble reactive phosphorus determination. The CV averaged 3.4% (range, 1.0 to 7.5%).

Counting of bacteria. Bacteria were fixed with 25% glutaraldehyde (final concentration, 0.5%), filtered onto Nuclepore filters (0.2-μm pore size; prestained with Irgalan black), stained with acridine orange, and counted by epifluorescence microscopy (14). Two or three replicate preparates were made for each culture. Solution blanks were checked each time new preparates were made. I counted 600 to 1,000 bacteria in at least 40 fields on each filter. The CV averaged 6.7% (range, 0.8 to 15.5%).

Biovolume determinations. For all biovolume determinations I used bacterial cells fixed with 25% glutaraldehyde (final concentration, 5%). The length (L) and width (W) of the cells were measured as described below, and the volume of the cells were calculated as $(\pi/4)W^2(L - W/3)$. This formula applies both to rods and cocci (L = W).

SEM. The fixed cells were filtered onto Nuclepore filters (0.2-μm pore size) and washed with filtered (0.2-μm pore size) distilled water. The cells were then dehydrated by covering the filters with a few milliliters of acidified 2,2-dimethoxypropane (DMP) (Sigma Chemical Co., St. Louis, Mo.) (24). The DMP was changed after 5 min by drawing most of it through the filter before adding more DMP. After dehydrating for 10 min (total time), the filters were carefully removed from the filter holder and placed in a small container with pure DMP. The dehydrated samples were critical point dried (CPD) by using liquid carbon dioxide (Critical Point Drying Apparatus, model E3000; Polaron, Watford, England). We found DMP to be mixable with liquid CO_2, and thus we omitted any transitional fluid. Pieces of the dried filters were mounted on aluminium stubs and coated with AuPd (60/40) (SEM Coating Unit E5100; Polaron). Coating was done for 3 min under the following conditions: 2.4 kV, 20 mA, and 0.05 torr argon atmosphere. To measure the thickness of the AuPd coating, I used latex spheres of known uniform diameter (0.25, 0.57, and 1.04 μm; Fluoresbrite Fluorescent Monodisperse Carboxylated Microspheres; Polysciences, Inc., Warrington, Pa.). The spheres were treated like the bacteria, but I omitted the dehydration and the CPD. The coating thickness was determined by comparing the transverse to the parallel diameter of linearly aggregated spheres (31). The coating thickness was found to be 32 nm (CV, 5.9%). This value was used to correct all length and width measurements. The coated samples were viewed in a JEOL JSM35 scanning microscope (accelerating current, 25 kV). Four to eleven photographs of each sample were taken at ×2,000 magnification on Kodak Verichrome pan film (125 ASA). The size of 60 to 190 bacteria from each sample was measured on photographic prints with a final magnification of ×6,000. The prints from each sample were remeasured two to three times to ensure repeatability of the results. The CV averaged 4.1% (range, 1.8 to 8.4%).

Acridine orange staining and epifluorescent microscopy. The samples were prepared as described for counting of bacteria, and the size of the stained bacteria was measured by using a calibrated eyepiece graticule (New Porton G12; Graticules, Ltd., England). This graticule has an array of 11 globes and circles with different diameters. The diameter increases with square root of 2 progression. With the magnification I used (×1,000), the smallest graticule circle had a diameter equivalent to 0.2 μm, and the largest had a diameter equivalent to 7.6 μm. The size of 50 to 70 bacteria was measured in each sample, and each sample was remeasured three to four times. The CV averaged 10.5% (range, 3.9 to 19.4%). Four to six photographs of each sample were taken on Kodak Ectachrome film (200 ASA). The size of 50 to 100 bacteria from each sample was measured by projecting the slides on a wall screen (final magnification, ×14,500). The photographs from each sample were remeasured three to four times. The CV averaged 6.9% (range, 3.0 to 10.5%).

Electronic particle sizing. The cell volumes were recorded with a Coulter ZB Channelyzer particle analyzing system connected to a Digital Eqiupment Corporation MINC laboratory computer. A 30-μm orifice tube was used. The samples were diluted in filtered (0.2-μm pore size; three times) 70% seawater. For calibration we used latex spheres with a diameter of 1.04 μm (same as for SEM). Each sample were measured 8 to 10 times. The CV averaged 6.0% (range, 5.3 to 6.6%).

RESULTS AND DISCUSSION

Bacterial carbon content and C/N/P molar ratios. The presence of capsules or extracellular organic polymers may be an important source of error when calculating the mass of dry matter, or of carbon, per unit of cell volume (1). As both capsules and extracellular polymers are mainly polysaccharides (7, 34), they may lead to an overestimated amount of carbon per cell, but the amount of nitrogen and phosphorus per cell will be unaffected. If extracellular products have contributed significantly to my carbon measurements, one would thus expect the N/C and P/C ratios I estimated to be low compared with the normal range of these ratios. I found the N/C molar ratio of the cells to range from 15 to 22% (Table 1). This range is within the range of N/C ratios published by others (22, 38).

The flexibility of bacterial cells with respect to phosphorus content is clearly demonstrated by the range of the P/C molar ratios (Table 1). The P/C ratios are, with the exception of the two extreme values found in the phosphorus-limited

TABLE 1. Comparison of cell composition, carbon per cell, estimates of cell volume obtained with different methods, and carbon per unit of estimated cell volume for six different cultures of bacteria

Inoculum	Limiting nutrient	Cell composition (C:N:P molar ratio)	g of C per cell ($\times 10^{-13}$)	Cell vol (μm^3) by:				C per unit of cell vol (10^{-13} g of C μm^{-3})[a] by:		
				SEM	Epifluorescence microscopy		Electronic sizing	SEM	Epifluorescence microscopy	
					Eyepiece graticule	Photographs			Eyepiece graticule	Photographs
P. putida	C	100:22:6.2	1.29	0.30	0.28	0.29	ND[b]	4.2	4.6	4.4
P. putida	N	100:19:5.1	1.69	0.39	0.66	0.71	0.74	4.4	2.6	2.4
P. putida	P	100:18:0.2	3.12	0.34	0.57	0.63	0.66	9.3	5.5	5.0
Mixed	C	100:21:13	1.06	0.14	0.11	0.19	ND	7.7	8.0	5.8
Mixed	N	100:15:4.9	2.14	0.24	0.30	0.48	ND	9.0	7.2	4.5
Mixed	P	100:16:1.8	1.91	0.32	0.27	0.55	ND	6.0	7.1	3.5

[a] The average values for SEM, eyepiece graticule, and photographs were 6.8, 5.8, and 4.2. The CVs for SEM, eyepiece graticule, and photographs were 32, 34, and 29%.

[b] ND, Not determined.

P. putida culture and the carbon-limited mixed culture, comparable to those found in the literature (unless explicitly given, carbon was assumed to be 50% [wt/wt] of dry weight) (22, 35, 38). The N/C and P/C molar ratios depend not only on the N and P content but also on the C content of the cells. Thus, the higher N/C and P/C ratios found in the C-limited cultures (Table 1) may be due to a low C content of the cells, as well as due to an increased content of nitrogen and phosphorus. The two extreme P/C ratios, 0.2% for the phosphorus-limited *P. putida* culture and 13% for the carbon-limited mixed culture, may be explained as the cooperative effect of a low P content and a high C content and vice versa, respectively.

By microscopic examination of negatively stained (India ink) samples, I was not able to demonstrate the presence of capsulated bacteria in any of the six cultures. As neither the N/C or P/C ratios nor the microscopic examination suggested that capsules, or any other extracellular material, were present in my cultures, I conclude that my carbon per cell values express the average carbon content of the bacterial cell bodies themselves.

The estimates of carbon content of cells range from 1.1×10^{-13} to 3.1×10^{-13} g of C $cell^{-1}$ (Table 1). Surprisingly, few measured values for carbon content per cell may be found in the literature. In fact, the only value I have found is 2.0×10^{-13} g of C $cell^{-1}$ for bacteria grown on *Laminaria* leachate (30). The authors, however, note that their estimated value is rather high even for bacteria in culture, and they proposed that the reason for such a high value might be the presence of large amounts of extracellular material.

The carbon-to-dry-weight ratio for bacteria is ca. 45 to 50% (4, 22). As this ratio is relatively constant I may compare my carbon per cell values with literature data on dry matter per cell. Dry-matter content ranging from 1.66×10^{-13} to 67.5×10^{-13} g of dry matter $cell^{-1}$ (average, 15.5×10^{-13}; $n = 17$) has been estimated for bacteria isolated from litter and soil (12). For *Escherichia coli* B grown under different conditions in chemostat cultures, dry matter has been reported to range from 1.33×10^{-13} to 3.77×10^{-13} g of dry matter $cell^{-1}$ (average, 2.3×10^{-13}; $n = 73$) (15).

From data on respiration rate, cell number, and growth rate, the masses of plate-counted bacteria and microscopically counted bacteria have been estimated to be 4×10^{-13} and 0.4×10^{-13} g of dry matter $cell^{-1}$, respectively (21). As the size of bacterial cells may vary considerably, it is not surprising to find a large range of values for the dry-matter content of bacterial cells. We find our carbon per cell values to be in reasonable agreement with published values.

Bacterial biovolume. Table 1 shows that biovolume estimates obtained by using different methods may vary significantly. The generally low CV values of the estimations do, however, indicate that the results obtained for a given culture with a given method are reproducible. Thus, the difference between the estimates does not seem to be due to errors in the estimates themselves. The reason for the difference may be found in the fact that cells were in different physiological states, that they were treated differently when preparates were made, and that cell size was measured by different methods.

Samples from all cultures were fixed in the same way. In the further treatment of the cells some were dehydrated and CPD, some were stained with acridine orange, and some fixed cells received no further treatment. Large and small cells, cells in different physiological conditions, and different types of cells (gram positive and negative, rods, cocci, etc.) may respond differently when exposed to the fixative and to the further treatment. It is impossible to predict how different cells respond to different treatments; however, the overall effect may be an unsystematic variation between biovolume estimates obtained with different methods.

To obtain accurate biovolume estimates from SEM photographs, it is important to correct the length and width measurements of the cells for the thickness of the AuPd coating (or any other coating used). This correction increases in importance with decreasing cell size. The volume of a sphere measured to have a diameter of 1.0 μm will be overestimated by 22% if the coating thickness is not corrected for (with the coating thickness assumed to be 32 nm; see above). The volume of a sphere measured to have a diameter of 0.5 μm will be overestimated by ca. 51%. Earlier investigators using the SEM method for estimating bacterial biovolume do not seem to have been aware of the importance of doing this correction (3, 8, 9, 23, 27, 43, 44).

The microscopic methods used for measuring the size of bacterial cells depend on proper judgment of the length and width of the cells. I used fluorescent latex spheres (see above) with different diameters to check how accurately cells of different diameters could be measured. When the diameter of the largest spheres (1.04 μm) was measured on SEM photographs and on photographs from the epifluorescence microscope, the results were in agreement with the known diameter of the spheres (Table 2). The diameters of

TABLE 2. Diameter and volume of latex spheres as measured by SEM epifluorescence microscope and electronic particle analyzer[a]

Latex sphere		SEM[b]		Epifluorescence microscope				Electronic sizing
				Eyepiece graticule		Photographs		
Diam (μm)	Vol (μm³)	Diam (%)	Vol (%)	Diam (%)	Vol (%)	Diam (%)	Vol (%)	Vol (%)
1.04	0.589	100	100	87	66	99	97	100[c]
0.57	0.097	96	88	88–123	68–186	91	75	ND[d]
0.25	0.008	88	68	80–120	51–173	84	59	ND

[a] The values are given as a percentage of the "true" values (supplied by the manufacturer).
[b] The values are corrected for the thickness of the AuPd coating (32 nm).
[c] The 1.04 μm sphere was used for calibration.
[d] ND, Not determined; the sphere size was below the limit of detection (see the text).

the smallest spheres (0.57 and 0.25 μm), however, were underestimated by both methods. When using the eyepiece graticule, I found the 1.04-μm sphere to match the 0.9-μm circle of the graticule. The 0.57-μm sphere matched between the 0.5- and the 0.7-μm circles, and the 0.25-μm sphere matched between the 0.2- and the 0.3-μm circles. Thus, the diameters of the two smallest spheres could easily be either over- or underestimated by ca. 20% (Table 2).

The 1.04-μm spheres were the only spheres large enough to be measured with the Coulter Counter. These spheres were also used for calibration (see above). The lower limit of detection of the Coulter Counter corresponds to particles with a diameter of ca. 2 to 3% of the orifice diameter (instruction manual for Coulter Counter model ZB). In this case, with a 30-μm orifice tube, this is ca. 0.6 to 0.9 μm, or equivalent to a sphere with a volume of 0.11 to 0.38 μm^3. Thus, only relatively large cells can be measured with this equipment. As any smaller cells in a bacterial population will not be included in such size measurements, the average cell volume of the total population may be overestimated.

The properties of the bacterial cells themselves, the treatment of the cells, and the methods used to measure cell size are of importance when estimating the bacterial biovolume. It is obviously very difficult to correct each single length and width measurement for any error that may be due to the specific treatment of the cell or the method used for measuring. As my main interest is the estimation of the carbon content of the cells, however, I am not interested in the native biovolume of the cells per se. Any systematic over- or underestimation of bacterial biovolume will automatically be compensated for by a correspondingly smaller or larger biovolume-to-carbon conversion factor.

Biovolume-to-carbon conversion factor. The carbon content per unit of cell volume (Table 1) shows a variation that is due to the inherent difference between the methods used for estimating biovolume, as discussed above. Superimposed on this variation there may be a variation that is due to the fact that different types of bacteria and bacteria grown under different conditions may have different carbon content per unit of cell volume.

When each of the three microscopic methods used for estimating biovolume was considered, I found that the average carbon content per unit of cell volume for the six different cultures had a CV of ca. 30% (Table 1). Due to this rather high CV value, there is no significant difference between the estimates obtained by the three different methods (Student's t test; significance level, 5%). An average biovolume-to-carbon conversion factor of 5.6×10^{-13} g of C μm^{-3} may thus be used for all three different methods.

The conversion factor I determined experimentally is 3.4 to 6.6 times higher than previously published theoretically derived conversion factors (3, 13). There are two reasons for this. The first is that theoretically derived conversion factors are often based on the assumption that the dry-weight-to-wet-weight ratio of bacterial cells is ca. 0.2 (3, 6, 39). As was shown in a previous paper (4), an average value of ca. 0.4 would be more reasonable for this ratio.

The second reason is that I may have underestimated the the native biovolume of the cells. This underestimation of the cell size may be due to a shrinkage of the cells caused by the use of fixatives, and in the case of the SEM method, also dehydration and CPD. The microscopic methods I used will also tend to underestimate the cell size (Table 2). The magnitude of the total underestimation is very difficult to estimate, as it requires that the native biovolume of the cells be known. Several authors have reported that bacterial cells tend to shrink when they are prepared for electron microscopy by dehydration and CPD. The amount of skrinkage reported ranges from less than 5% to more than 40% in length or width of the cells (3, 8, 23, 26, 36, 42). However, important factors, like the effect of the use of fixatives, the thickness of the AuPd coating, and the accuracy of the method used to estimate the native biovolume of the cells, have in most cases not been considered. One should therefore be very careful when using literature data to correct for any apparent shrinkage of the cells. If I assume that I have underestimated the length and the width of the cells by 25%, I will have estimated a biovolume of the cells that is only 42% of the native biovolume. When my biovolume estimates are corrected for this amount of underestimation, I find that the specific carbon content of the cells is 2.4×10^{-13} g of C μm^{-3} (average for all methods). This value agrees well with the value of 2.2×10^{-13} g of C μm^{-3} which I calculated from a dry-weight-to-wet-weight ratio of 0.4, a carbon-to-dry-weight ratio of 0.5, and a density of 1.1 g cm^{-3} (4).

The obvious consequence of my findings is that previously published data on bacterial biomass based on biovolume measurements (3, 6, 9, 13, 20) may be underestimated by a factor of ca. 5. When bacterial biomass production is estimated from the frequency of dividing cells (13, 20, 27) or thymidine incorporation (2, 9, 26, 29) and measurements of bacterial biomass, the production may also be underestimated by a factor of ca. 5.

Based on different methods, some workers claim the bacterial growth yield or conversion efficiency to be in the order of 60% (for a review, see reference 41), whereas others claim it to be in the order of 10% (for a review, see reference 25). A high value is found when the conversion efficiency is calculated from data on the amount of ^{14}C-radiolabeled substrate incorporated and respired. A low value is found when it is calculated from data on the amount of bacterial biomass (as carbon) produced and the amount of substrate (as carbon) consumed. However, as the bacterial biomass in the latter case is estimated from biovolume measurements, it may be underestimated by a factor of ca. 5. If this is taken into consideration, it will bring the conversion efficiency obtained with this method to the same order of magnitude as the conversion efficiency obtained with radiolabeled substrates. Thus, when microbial food webs are the concern, my findings support the view that bacteria play the role of secondary producers rather than mineralizers.

When bacterial biomass production is estimated, it is in

general found to be in the order of 5 to 25% of the primary production (9, 10, 13, 20, 30). If this bacterial production is underestimated by a factor of 5 and the bacterial growth yield is assumed to be 50%, the bacteria will consume 50 to 250% of the total fixed carbon. Although bacterial consumption may exceed the primary production on a short time scale (hours, days), this is not possible on a longer time scale (years). I might therefore argue that my finding indirectly supports the view that the $^{14}CO_2$ tracer technique seriously underestimates primary production (11, 16, 28, 32, 33, 40). On the other hand, however, I might on principle also question the methods used for estimating bacterial growth rate. If the growth rate is overestimated, the bacterial production and consumption will be overestimated.

Any method involving the use of a conversion factor will in most cases have to rely on the assumption that the conversion factor used is valid for the system under investigation. Due to the lack of methods, I have not been able to check the assumption that the conversion factor I derived from data on cultured bacteria is valid for bacteria as they appear in nature. However, as my conversion factor is derived from direct measurements of bacterial cell carbon content, cell number, and biovolume, I believe that it is more reliable and more easy to verify than any theoretically derived conversion factor. I would thus suggest that the value of 2.2×10^{-13} g of C μm^{-3} may still be used to estimate carbon content of bacteria on the basis of known or estimated native biovolume of living cells (4). If a factor for the conversion of microscopically measured biovolume of fixated bacterial cells is called for, a value of 5.6×10^{-13} μm^{-3} should be used. It should be realized, however, that the reliability of this last conversion factor is dependent on how the cells are treated and how the biovolume is estimated.

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