

Effect of nutrient kinetics and cytoarchitecture on bacterioplankton size

Abstract—Expanded specific affinity theory specifies the advantages of small dry mass and dilute cytoarchitecture in impoverished systems. For marine samples, bacterioplankton mean dry mass, according to flow cytometry, was near 24.5 fg cell⁻¹. Conversion to volume with buoyant density gave a cell volume of 0.124 μm³. Total DNA was 2.9 fg cell⁻¹. This compared with the size of a single genome of a small extinction-culture isolate, *Sphingomonas* sp. RB2256, of 3.96 fg or 3.6 Mb. The genome size of such isolates and other cultures decreased with metabolic simplicity. It was found that bacterioplankton could be exposed to radiolabeled amino acids and then sorted for size and that the specific affinities of the fraction of small organisms were as great as the fraction of large cells. Size, DNA, and metabolic-complexity distributions were concordant with the concepts that cell volume approaches a minimum set by sufficient space for the smallest genome that can provide sufficient information for competitive dissolved-nutrient acquisition, and that space requirements are further alleviated by the expression of few cytoplasmic-enzyme molecules in each of the various pathways and a dilute cytoplasm. Bacterioplankton approached a minimum genome size of 1.7 Mb with a minimum cell volume of about 0.06 μm³ and a DNA content of 16% dry weight. The property of small dry mass with a low DNA content was common in in situ bacteria but absent from cultivated representatives, which led to speculation that failure to grow in the laboratory is related to missing regulatory information.

The major roles of bacterioplankton in remineralization (Carlson et al. 1998) and food webs (Andersen and Fenchel 1985) are well known and based on only relatively recent visualization (Daley and Hobbie 1975). Small size, resistance to cultivation (Button et al. 1993), and low activity have hampered veridical characterizations. The environment of these transtrophic (nonpredatory heterotrophic or surface feeding) organisms is dilute, and yet they must concentrate sufficient organics from it for sustenance. Quantitative and mechanistic descriptions of the associated kinetics have been difficult because neither cell size nor permease content is easily incorporated into the Michaelis-Menten paradigm, even for more easily characterized organisms.

Here we examine the effect of enzyme-permease ratios on rate and saturation constants for nutrient uptake as specified by specific affinity theory (Button et al. 1998). The theory is used to explain the small size and dilute nature of oligobacteria as based on the distributions of cell mass, DNA content, and genome sizes. Flow cytometry is employed to quantitatively define these distributions and to examine the rates of nutrient acquisition by natural populations of large and small cells prior to physical sorting by the cytometer.

Kinetic theory and assumptions—Nutrient collection ability for intact cells can be specified by their specific affinity and

$$v = a_s X S, \quad (1)$$

where v is substrate uptake rate, a_s is the second-order whole-organism rate constant or specific affinity, X is biomass, and S is the concentration of substrate. Units of biomass concentration⁻¹ time⁻¹ easily accommodate nutrient flux, and change in molecular weight of the substrate is facilitated by use of grams rather than moles. Other properties of the microflora, such as total carbon or surface area, might be used in place of wet weight, but some quantitative indication of the amount of active cell material is necessary. Both saturation and the ability of cells to accumulate substrate are traditionally specified by a Michaelis constant K_m . This kinetic constant derives from the ability of an enzyme or permease to bind rather than transport substrate, as based on the Langmuir absorption isotherm as adapted to enzymology, and is a relative parameter based on the maximal transport activity V_{\max} of the organism-substrate combination. Specific affinity theory has the maximum or base value of the specific affinity a_s^0 dependent on the number N_p of permease or initial enzyme molecules such as P in the metabolic sequence catalyzed by, for example, proteins P_1 to E_4 (Fig. 1). Maximal substrate collection efficiency, and thus a_s^0 , is set by the frequency of molecular collisions of S with N_p and is achieved only at low substrate concentration. For hyperbolic saturation, a_s asymptotically approaches zero as v approaches V_{\max} with increasing S

$$a_s = \frac{N_p c}{1 + S c \tau} \quad (2)$$

where τ is the residence time of the substrate on P and c is a collection constant dependent on the size and properties of the cell and the substrate.

Assumptions are that the cell is spherical, that collection efficiency is limited by the total area of active site for the first catalyst of the sequence such as P_1 , the cells are energized so that all unoccupied active sites are receptive, and that members of the enzymatic pathway are connected so that τ is a composite function of their catalytic constants k_{cat} . If τ is taken as the reciprocal of the catalytic constant k_{cat} for the permease: $k_{\text{cat}} = V_{\max} N^{-1}$, V_{\max} is eliminated as a specific value for the asymptote. Thus the effective value of τ may change as nutrient flow from the permease to final macromolecule or other product varies with kinetic, thermodynamic, or regulatory pressure in response to intercellular and intracellular concentrations. The shape of the kinetic curve can then become any monotonic function of the concentration of limiting nutrient.

Advantages of Eq. 2 are (1) that the affinity of the cell for substrate can be related to the permease content of the cell, (2) that the value of the affinity constant for saturation K_a can be measured in the absence of V_{\max} data, which may be indeterminate for oligotrophs, and calculated from permease or enzyme concentrations and their catalytic constants, (3) that the shape of the kinetic curve specified by Eqs. 1 and 2 becomes infinitely flexible and suitable for

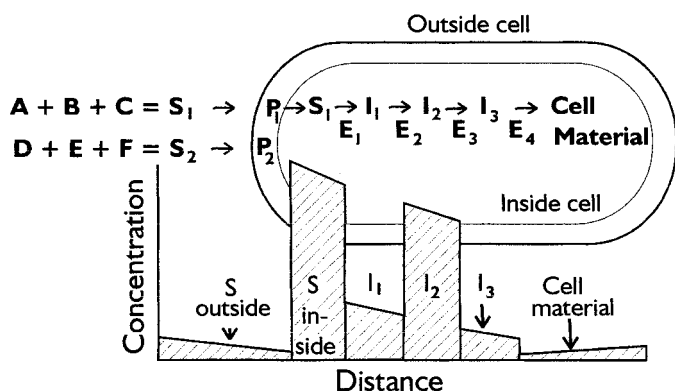


Fig. 1. Model of the uptake of two groups of nutrients S_1 and S_2 by a cell showing accumulation via their respective permeases P to be metabolized by a series of enzymes to various intermediates I and cell material. Concentration changes along the sequence are suggested.

accommodation of extended sequences as intended by Monod rather than dependent on the Michaelis Menten derivation, and (4) that the kinetic constants can become a measure of τ as controlled by various system components rather than by the frequency that collisions are reflected from an active site.

Permease-enzyme ratios—A system low in cytoplasmic enzyme may have a large a_s° due to a large permease content, but the saturation constant can be small as the few cytoplasmic enzymes become occupied with intermediates I at external substrate concentrations that are also small. Thus, τ transcends from a value set by the catalytic constant of the permease, the in sensu interpretation of Michaelian kinetics in microbiology, to residence time along the whole pathway. This parceling out of control through the effective values of both τ and N is the basis of the flux-control theorem (Brown and Cooper 1994). For example, if there are only a few molecules of E_3 or if E_3 has a small catalytic constant, the permease can build large concentrations of S_1 thermodynamically favoring production of I_1 . But the resulting large I_2 concentration will accelerate flow through E_3 to give a good supply of I_3 for cell material and amelioration of bottleneck limitation of flux by E_3 . N still controls a_s° since the restriction has no effect on the cell's ability to accumulate substrate at low concentration. At high concentrations, the flow to cell material is restricted by the few E_3 molecules, thereby reducing maximal rate. Since all members of the pathway are connected, the effective value of τ for the permease τ_p is increased when connected enzyme molecules are few. A material balance at steady state gives

$$\tau_p = \tau_E \frac{N_p}{N_E} \quad (3)$$

where N_E is the number of molecules of enzyme E_3 . But so long as the ratio $N_p:N_E$ is insufficient to effect significant increase in τ_p above its characteristic value, a_s° remains unchanged. Only the saturation-dependent value a_s is lowered. The affinity constant is therefore increased and rates are increasingly subject to saturative inhibition if substrate con-

centrations rise. Macromolecule biosynthesis rate at E_4 might dominate control as well, but environmental nutrient concentrations are low, so associated slow steps may be less influential. Taken in toto, the number of cytoplasmic-enzyme molecules per enzyme type can still affect major control on uptake rate, but only at substrate concentrations that are large. For oligobacteria, cytoplasmic enzymes can represent an excess energy sink to be minimized. These kinetics are termed janusian (Button 1991) because the permease sets the rate of transport following a "look" at the concentrations in both the forward and reverse directions.

Kinetic constants— N and τ may not be known, but a_s from Eq. 2 when substituted into Eq. 1, is given by vS^{-1} when rate (velocity v in the vernacular) is defined in terms of cell mass, and the value holds for all concentrations of substrate. Then a_s° is the intercept of a vS^{-1} versus v Eadie-Scatchard or affinity plot, and the reciprocal slope at the low concentration end of a Lineweaver-Burk rearrangement. At higher concentrations, saturation reduces a_s° to the rate constant a_s where a_s is analogous to $V_{\max}(K_s + S)^{-1}$ from the Michaelis Menten equation with a K_m of S at $V_{\max}2^{-1}$. The affinity constant $K_a = Sc\tau$ resembles K_m except that $K_a (= S \text{ at } a_s^\circ 2^{-1})$ is compared to initial slope rather than maximal rate V_{\max} . K_a is the concentration that gives the second term in the denominator of Eq. 1 a value of unity and can therefore be resolved from first principles. K_a is smaller than K_m , where saturation remains incomplete at high concentrations of substrate; i.e., τ (and τ_p where controlled by downstream enzymes) increases with S . Concordant with extensive homology among permeases (Griffith and Sansom 1998), large binding constants are not a necessary nor even useful requirement to attain the high affinities needed for the growth of pelagic bacteria, contrary to what might be inferred from formulations for isolated enzymes. Their high affinities, taking all utilized substrates in concert, may simply be associated with a large permease content. The size of the saturation constants, both K_a and K_m , is dependent on the effective permease:cytoplasmic-enzyme ratio (Eq. 3). When cytoplasmic-enzyme content is low, K_a is reduced, but this does not mean high affinity, and the specific affinity a_s° remains unchanged. Oligobacteria should therefore be predisposed to small affinity constants and high affinities, not because of unique permeases, but because of dilute cytoarchitecture.

Multiple substrates and enzyme content—The ability of bacteria to sequester organics is improved by using several substrate types simultaneously. Flux of a single nutrient type is thought to be maximal with only sparse spacing of permeases due to restrictions imposed by a collisional limit, and surface area becomes available for collecting additional types without slowing transport of any single substrate species (Button 1998, and references therein). Numerous permease types are required to transport this diversity of species while retaining sufficient specificity to exclude harmful members. Permeases have some breadth in specificity, so that one species, for example A , might be transported by P_1 , a permease or protein that is sufficiently nonspecific to include some other substrates B, C, \dots defined as a group with a total concentration of S_1 . Neglecting interactive ef-

fects, such as those mediated by membrane potential with a second group S_2 ($= D, E, F$) that is transported with affinity a_{S_2} , the flux of the separate species is given by the associated affinity, individual substrate concentrations, and total biomass. For a group such as S_1 the substrate concentrations comprising S_1 should be additive with flux given by the product of the associated specific affinity and total concentration of the group. Intragroup components such as B and C would reduce specific affinity a_{S_1} at saturating concentrations but members of S_2 should not. The amount of cytoplasmic enzyme E_3 should therefore be just sufficient to avoid significant saturation by intermediates from any of the S_1 pathway members at the concentrations of substrate encountered in the environment; more incur the costs of additional enzyme synthesis, regulatory proteins, and DNA.

Specific affinity and cell size—In the above model, shape is taken as spherical for simplicity and is a good approximation for many small morphotypes, although shapes with higher surface-to-volume are common and a few appear to have long thin straight fibrils appended. For the spherical shape, cell size is given by the radius r_x and c where $c = 5DMr_s^2(2r_x^4)^{-1}$, D and M are the diffusion constant and molecular weight of the substrate, and r_s is the effective radius of the active site. With cell radius in the denominator, specific affinity can be larger in cells that are small. Also small organisms are less valuable to predators, so minimal size becomes doubly advantageous. Although specific affinity is increased by the use of multiple substrates because the concentration at the collisional limit for the combined resource is increased, use of multiple substrates to achieve oligotrophy incurs the cost of an increased genome size.

Space for the genome can be accommodated by cells with few cytoplasmic enzymes if they are dilute. Diluteness at 18 to 19% dry matter is a property of both isolates *Cycloclasticus oligotrophus*, and *Marinobacter arcticus* (Button et al. 1998), the only marine bacteria with known refractive indices and buoyant densities. So the small affinity constants of marine bacteria can, but do not necessarily reflect high affinity. Diluteness and small size, both of which give a large surface to dry-mass ratio, may be the result of evolutionary forces to increase nutrient acquisition rates from nutrient-depleted systems.

In situ cell mass—The distribution of dry-mass content among organisms up to about 1- μm diameter in a typical seawater sample according to flow cytometric measurements (Button and Robertson in prep.) is shown in Fig. 2. Water was an oceanic sample from 1.5 meters in the Gulf of Alaska in May. While there was the expected decrease in total population below the euphotic zone, the range in mean mass per cell was only 21 to 24 fg cell⁻¹ over 150 m (in prep.). Euphotic zone values over 4 yr, May–August, were 24 fg cell⁻¹, sd = 2, $n = 5$ including estuarine locations (in prep.). These small values for dry cell mass (Table 1) compared with those recently reported for oceanic bacteria of 24 and 60 fg cell⁻¹ by combustion analysis of oceanic and coastal waters (Fukuda et al. 1998), and with the commonly used values of 40 fg (20 fg C) per cell (Psenner 1990) used in productivity calculations. The median cell volume of 0.094 μm^3 com-

pared with literature values of from 0.018 to 0.19 μm^3 (Loferer-Krössbacher et al. 1998; Sherr et al. 1992; Sieracki et al. 1985; Viles and Sieracki 1992), a large range in both cases that appears to reflect mostly difficulty in characterizing the physical properties of oligobacteria.

Genome size and DNA content—Values for pure cultures can be determined by the DAPI (4',6-diamidino-2-phenylindole)-DNA fluorescence of single cells (Robertson et al. 1998) using standards of equivalent salinity (Button and Robertson in prep.), where standard curves gave $r^2 = 0.99999$ ($n = 5$) without intercept, and there was agreement with independent determinations by wet methods, but correction for GC content is required. By this technique, for example, the otherwise unknown genome size of *Sphingomonas* sp. RB2256, an extinction-culture isolate (Fegatella et al. 1998) described as an ultramicrobacterium (Schut et al. 1993), is estimated from the 1 n DAPI-DNA fluorescence using a GC content of 65 mol% (Segers et al. 1994) to be 3.9 Mb (Fig. 3).

Most marine bacteria have a high fraction of genetic material in their dry matter due to small cell size. For indigenous forms, DNA was 12 to 16% of their dry weight (Table 1). The data could include small *Prochlorococcus* cells with chlorophyll-quenched DAPI fluorescence, but none were apparent in these high-latitude waters. Heterotrophic bacterial cells tend to be more spherical at the smaller end of the size range than for the bulk population where a 3:1 axial ratio was used in computing mass. This would give a 20% underestimate in the mass of cells having a volume of 0.1 μm^3 (Robertson et al. 1998), and their DNA content would be overestimated accordingly. AT-rich (low GC) cells will also appear high in DNA content due to preferential DAPI binding. Most morphologies approached the prolate spheroid assumed, and the GC content of most rRNA-DNA is near the 52% used here to estimate the DNA content of indigenous forms (Fig. 2).

The DNA content of the total heterotrophic bacterial population, at 2.94 fg cell⁻¹, gives a much higher percentage DNA than the 1.1 to 4.4% dry weight of those commonly cultured. For the depth distribution mentioned above, the range was only 1.8 to 2.9 fg cell⁻¹, minimum near the base of the euphotic zone, and typical of our seawater measurements. Literature values for seawater of 8.1 to 14.5 fg-DNA cell⁻¹ (Paul and Carlson 1984) are near the large values obtained for most commonly studied bacteria, but according to the cell-mass measurements for indigenous forms here, a perhaps unreasonable DNA content of near 50% would be required to achieve this composition.

DNA in the distribution shown (Fig. 2, note logarithmic scale) contained peaks at 1.4 and 2.2 fg cell⁻¹, and there was an additional cluster of large cells at 7 fg. The DNA content of cells can increase with growth rate due to additional replication forks. Increased content can therefore reflect increased rates of growth, such as found in the euphotic zone, but other factors, such as chromosome runout initiated by starvation to obtain the DNA standard curves used here, can complicate the DNA content/activity relationship.

Attempts to obtain typical bacterioplankton representatives used extinction (extensively diluted) culturing in un-

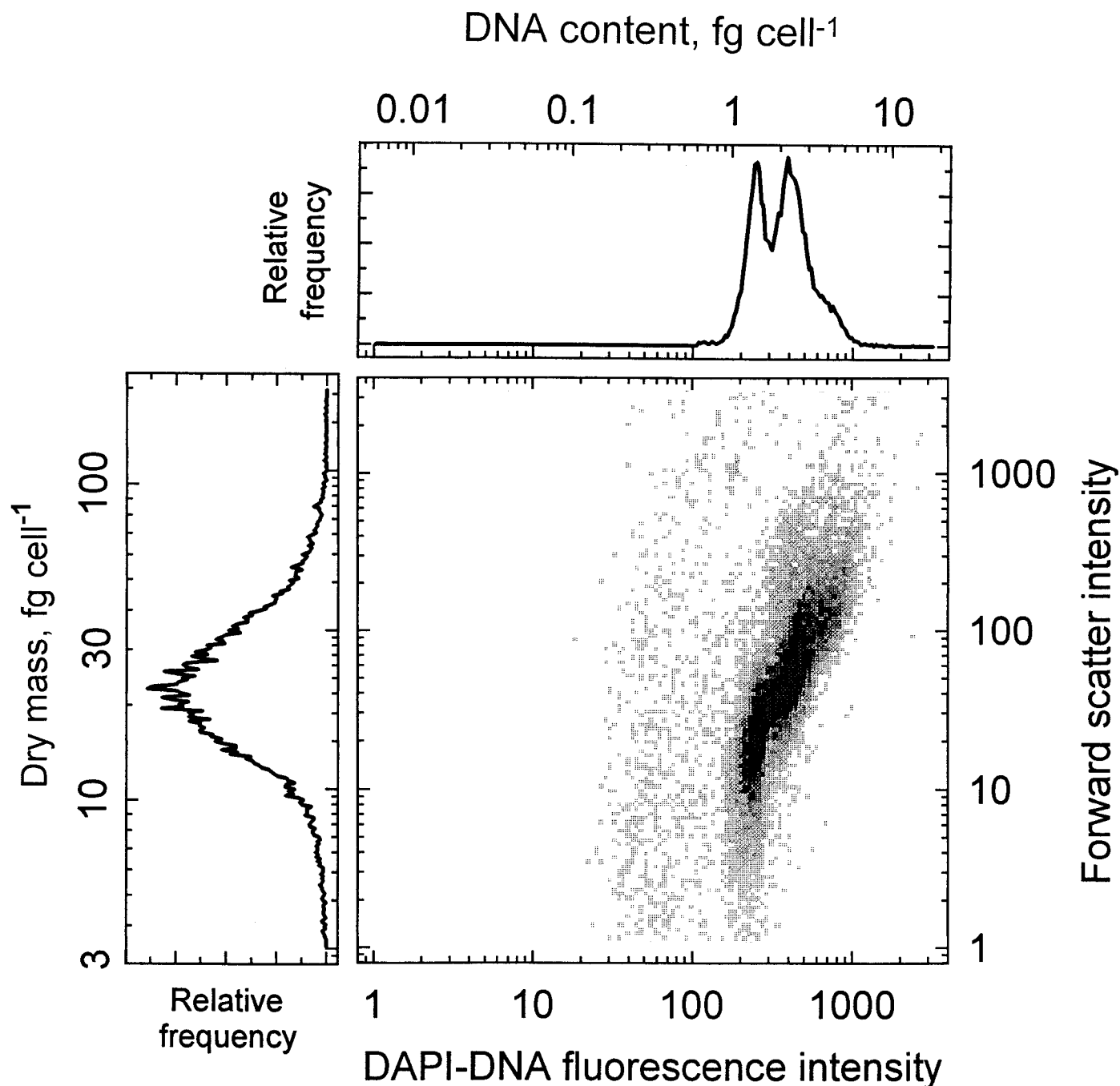


Fig. 2. Distribution of dry mass and DNA content of the organisms in a 1.5 m seawater sample. Side bars collect the separate parameters as frequency histograms.

amended seawater to minimize exogenous substrate. Isolates obtained included *Cytoclasticus oligotrophus* RB1 (Button et al. 1998) and *Sphingomonas* sp. RB2256 (Fegatella et al. 1998). The DNA content of their dry material was 10.7 and 11.1% respectively, and near the 12% DNA found in bacterioplankton (Table 2). The range in DNA at the extreme values in size in these indigenous forms was a very narrow 13 to 16%. That the minimum value is at the mean for size is consistent with higher values for the small cells due to size limitation by DNA content and consistent with the high

values for the large cells due to the presence of multiple genomes.

When the genome content of *C. oligotrophus* was increased from one to five by substrate deprivation, there was a 65% rather than fivefold increase in the fraction devoted to DNA (Button and Robertson pers. comm.), consistent with an increased space requirement for the additional DNA. Dry mass is generally smaller for in situ bacteria than for marine and other isolates. We are able to grow only 3 to 10% of the bacterioplanktonic organisms by extinction cul-

Table 1. Dry mass and DNA distributions of $<1 \mu\text{m}^3$ organisms from 1.5 m in the Gulf of Alaska.

Statistic	Total solids (fg cell ⁻¹)	Volume (μm^3 cell ⁻¹)‡	DNA content	
			(fg cell ⁻¹)	(% dry wt)
Mean	24.5	0.124	2.94	12
Median	18.5	0.094	2.66	14
Mode	18.5	0.094	2.92	16
Small limit*	10.5	0.054	1.66	16
Large limit†	35.0	0.178	4.54	13

* Maximum mass of the lightest 5% of the 19,700 particles analyzed from a population of $1.91 \text{ million ml}^{-1}$ population characterized in Fig. 2.

† Minimum mass of the most massive 5% of the particles.

‡ Assuming a solids content of 18.5% and a buoyant density of 1.063.

turing, and cultivation of members of the prevalent 1.7 Mb bacterioplankton is yet to be described, so knowledge of the cytoarchitecture of in situ forms is limited. A few organisms with smaller genomes are suggested by Fig. 2, but apparent values could be underestimates due to species with a low content of the DAPI-sensitive AT-triplet base pairs.

Effect of size on activity—It is argued that many of the small cells may be cytoplasmless (Zweifel and Hagström 1995) or DNA-free (Tabor et al. 1981) ghosts due to phage infection (Wilhelm et al. 1998) that give reversible staining (Zweifel and Hagström 1995) or include normal cells dwarfed by oligotrophic conditions to increase the surface-to-volume ratio. However, the specific affinity for ¹⁴C-mixed amino acids by filter-fractionated bacterioplankton was nearly constant for the various populations and maximal for those small forms passing through 0.6- μm pores (Button and Robertson 1989).

This finding of high activity in small cells was reinvestigated by exposing a population to labeled amino acids, sorting the organisms by flow cytometry according to size and DNA content, measuring the label incorporated, and determining their specific affinity. Surface water was collected from Harding Lake 60 km south of Fairbanks in June when activity, according to specific affinity for amino acids, was high, flown to the laboratory in an insulated 20-liter carboy, amended with $1 \mu\text{g liter}^{-1}$ of the amino acid mixture 7 h later, and incubated for 3 h. Samples were separated into fractions containing equal mass but comprised of either small, low-DNA organisms or ones that were larger with more DNA (Fig. 3), and the radioactivity of the fractions was determined by scintillation counter. Sorting and analyses here were with an Ortho operating system-equipped cytometer (Robertson and Button 1989). Reanalysis of the sorted fractions indicated that the small and large organisms were completely separated, giving only those organisms with the desired properties in each fraction as shown. Radioactivities of the unsorted cells, specific affinity 353 liters g cells wet wt⁻¹ h⁻¹ for the amino acid mixture, were unchanged by the 100-fold dilutions with the cytometer sheath fluid associated with sorting (Table 2). Neither was there a significant difference between the mass-based activities of the cells in the large- and small-cell fractions. Data show that large numbers of bacteria can be sorted with fidelity by flow cytometry.

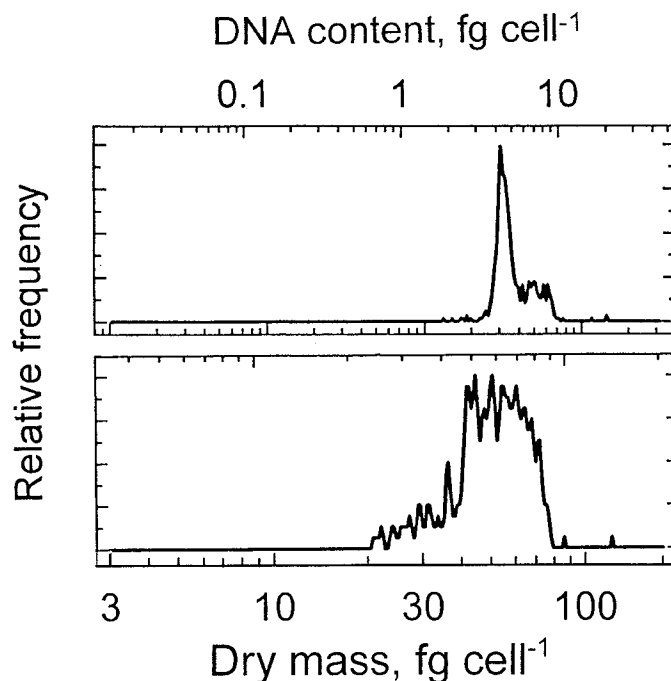


Fig. 3. Mass and GC-corrected DNA content histogram of extinction-culture isolate *Sphingomonas* sp. RB2256. Genome size was taken from the large or B-phase peak.

Autoradiography typically indicates that about half the cells are able to accumulate a single substrate such as leucine in active waters with no obvious size discrimination (Button and Robertson in prep.) consistent with the sorting results above and with the data of Ouverney and Fuhrman (1999). Starved bacterial cultures such as *C. oligotrophus* quickly lose about 90% of their DNA but not their dry mass (Robertson et al. 1998), and are thus recognizable (Figs. 2 and 4A). Such dim cells (located to the left of region 1) are particularly prevalent in active waters (Button et al. 1996), can be generated in the laboratory, and seem to be less abundant in more oligotrophic conditions such as those associated with Fig. 2, perhaps related to a slow production rate. Expired cells would appear in the small fraction if they were to lose part of their DNA following virus infection because of the grouping of small size with low DNA. As such they would dilute activity of the small-cell fraction and cause it to appear less active than the large. Either the moribund organisms had a negligible effect, or the living small cells

Table 2. Amino acid uptake rates by small, low-DNA freshwater bacteria, as compared to large, high-DNA organisms.

Fraction	Histo-gram region*	DNA mean, (fg cell ⁻¹)	Dry mass mean (fg cell ⁻¹)	Uptake, ag amino acids (pg wet h) ⁻¹ †
Unsorted	3	1.6	12.7	141 ± 14
Small	1	1.2	9.0	146 ± 19
Large	2	3.1	33.2	129 ± 21

* Data taken from Fig. 4.

† Rates based on the uptake rate measured in two separate experiments.

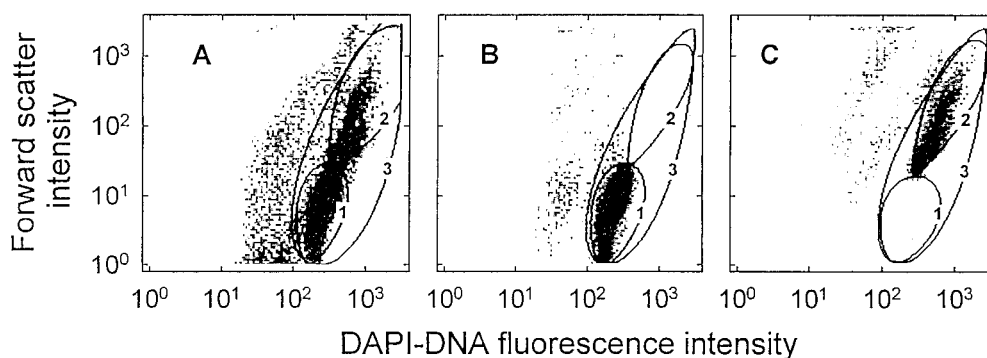


Fig. 4. Sorting of lakewater bacterioplankton by flow cytometry following exposure to radiolabeled amino acids. (A) Total particles, bacteria selected (region 3); small, low-DNA organisms region 1; large, high-DNA organisms (region 2). (B) Reanalysis of cells sorted from region 1. (C) Reanalysis of cells sorted from region 2.

had an even larger activity than the big ones. Consistent with the predictions from theory, evidence suggests that small low-DNA bacteria of seawater have specific affinities that are at least as great as the large bacteria.

DNA and cell size—Considering both bacterioplankton and cultivars (Fig. 5), genome size (total DNA in the case of bacterioplankton) and cell mass are related. Genome size increases with dry mass from a threshold value to a value approximately three times larger where cell size can increase further without burdening the organism with additional DNA. Nutritionally restricted organisms such as *Brevundimonas diminuta* (Segers et al. 1994) and *C. oligotrophus* use only a few substrates, whereas more versatile organisms such as *E. coli* use more and sustain the genes for hundreds of permeases (Paulsen et al. 1998). These restrictions may help facilitate the small genome-size of *B. diminuta*, while its more robust morphology is consistent with the additional cytoplasmic-enzyme content required for competition at higher substrate levels. Viabilities determined by extinction

culture are lower with added substrate (Button et al. 1993), perhaps due to restricted regulatory capability in these small-genome organisms. Successful competition in dilute environments appears to require a genome size for free-living bacteria of at least 1 fg cell⁻¹, with much more than 2 fg DNA becoming burdensome. RNA is minimized as well, and rRNA operons are reduced to one (Fegatella et al. 1998). If *M. arcticus* and *C. oligotrophus* are typical, then indigenous marine bacteria are dilute as well as small as expected from theory. Diluteness allows maximal permease content for a given dry mass and space for replication of the rigid (Daune 1999) DNA molecule and for changes in tertiary structure, gyrations that are thought to function in regulation (Barry et al. 1992). Sustained high affinity can be achieved with few cytoplasmic enzymes so long as they are sufficient to facilitate flow at the small ambient substrate concentrations present. At 19% dry weight, 16% of which is DNA, the minimum size of a 1.7 fg DNA organism is 0.06 μm³. Although smaller organisms may exist in a rich environment (Carson 1998) 0.06 μm³ appears to be near the minimal size for aquatic bacteria. The theoretical advantage of a high permease to dry-mass ratio, the abrupt absence of free-living bacteria below 1 fg in DNA content, the increase in size of *C. oligotrophus* with DNA content, the increase in genome size with nutritional complexity, and the equality of uptake rates by small and large bacterioplankton are all consistent with the concept that bacterioplankton arrive at a minimum volume that is set by space requirements for their DNA at the minimum level of complexity consistent with successful competition. These forces combine to give point sources for remineralized organics in ways that combine to maintain exceedingly pure aquatic systems in terms of reactive dissolved organics.

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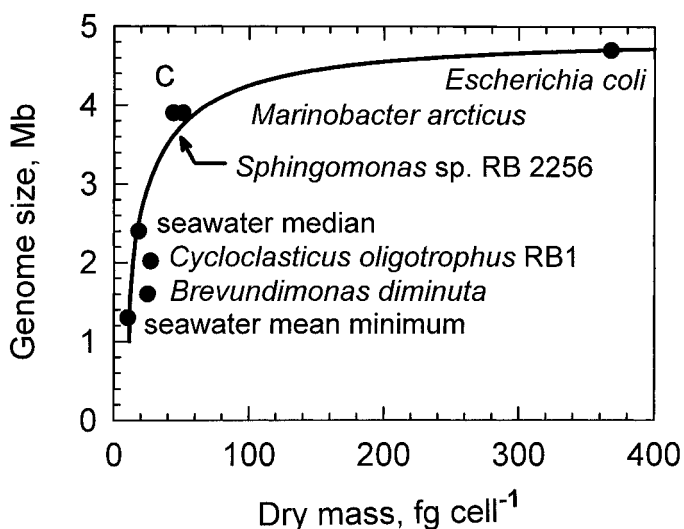


Fig. 5. Relationship between genome size and dry mass for bacterial isolates taken from data such as shown in Fig. 3, with values for indigenous organisms from Table 1.

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