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NOTE

Variation of carbon content among bacterial species under starvation condition

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ABSTRACT: To explore the variability of bacterial biomass conversion factors in aquatic environments, 5 marine and 5 non-marine bacterial strains were exposed to nutrient deprivation in artificial seawater and physiological (NaCl, 9 g l^{-1}) water, respectively. Carbon content per cell (C_c) and per volume (C_v) were estimated from total cell number, particulate organic carbon and cell volume (V) measurements after 0, 7, 15 and 28 d of starvation. While C_c appeared to be rather constant (26.02 ± 1.08 fg C $cell^{-1}$; mean ± standard error, n = 50) during starvation, C_v showed a significant increase. In general, C_v increased more rapidly in marine strains than in non-marine strains. C_c and V were not correlated, but a highly significant relationship between C_v and V was found. The non-linear relation was well fitted by a power function model (r^2 = 0.847, $p < 0.0001$). Parameters of the power function adjusted to marine or non-marine strains were not significantly different. We conclude that C_v and, to a lesser extent, C_c were dependent on species and physiological status. However, it seems that the differences in C_v between marine and non-marine bacteria observed in this study and by others are not the consequence of different relationships between C_v and volume, but, more likely, of the low cell volume of marine bacteria and their ability to reduce their water content faster than can non-marine bacteria.

KEY WORDS: Bacteria · Carbon content · Starvation

To understand the trophodynamics or biogeochemical role of bacteria in aquatic ecosystems, several studies have focused on conversion factor estimation to transform bacterial abundance or biovolume into biomass. The reported values of carbon content per cell (C_c) or carbon content per volume (C_v) exhibit a large range of variation (e.g. see Ducklow & Carlson 1992, Fagerbakke et al. 1996).

Such a large variability may be partly explained by the difficulty of obtaining precise and reproducible biovolume measurements (Nagata & Watanabe 1990). However, carbon content per cell values, which are not dependent on volume estimation, also show large variations. Several hypotheses have been proposed to explain the variation of the conversion factors: (1) differences in the taxonomic composition of bacterial communities, which may depend on sampling time and location (Nagata & Watanabe 1990, Kroer 1994), and (2) differences in physiological states of bacterial cells (Nagata 1986), which may be related to the trophic status of the ecosystem. However, to our knowledge, these hypotheses have never been explicitly tested.

C_c and C_v values estimated from bacterial cultures are scarce (see Fagerbakke et al. 1996) and have been obtained from cells grown under rich nutritional conditions (culture) which are very different from oligotrophic conditions of marine waters (Roszak & Colwell 1987). Low ambient substrate concentrations induce bacterial cell starvation, in particular resulting in cell size decrease (Morita 1993). This phenomenon seems to be experienced by a large number of bacterial cells in aquatic ecosystems, especially in the oceans (del Giorgio & Scarborough 1995). So, as outlined by Norland (1993), it is important to know if the relationship between biomass and biovolume (C_v factor) can be described by a constant ratio or if it exhibits a size dependence.

In the present study, we measured C_c and C_v values for 5 marine and 5 non-marine strains exposed to nutritional deprivation, to evaluate (1) the differences in conversion factors among different bacterial strains, (2) the effect of starvation conditions on these factors, and (3) the relationship between these factors and cell volume.

Materials and methods. ***Bacterial strains and starvation conditions:*** The 5 bacterial strains of marine origin

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(*Alteromonas nigrifaciens*, *A. rubra*, *A. tetraodonis*, *Vibrio campbellii* and *V. fischeri*) were obtained from P. Lebaron, Laboratoire Arago, Banyuls sur Mer, France. The 5 strains designated non-marine bacteria (from enteric or freshwater origins) were obtained from the ATCC (*Aeromonas hydrophyla* ATCC 15467, *Escherichia coli* ATCC 14948) or were from our collection (*Enterobacter cloacae*, *Pseudomonas putida*, *Salmonella typhimurium*) and previously isolated from freshwater in the Montpellier (France) area.

Marine strains were maintained on marine agar 2216 (Difco, Detroit, MI) at 20°C. Other strains were maintained on nutrient agar (bioMérieux, Marcy l'Etoile) at 30°C.

Before each assay, colonies from marine or non-marine strains were transferred to marine broth (marine broth 2216, Difco; 20°C) or to trypcase-soy broth (bioMérieux; 37°C), respectively, and incubated for 24 h (stationary phase).

The cultures were then harvested by centrifugation at 1000 × *g* for 10 min, washed twice with either sterile artificial seawater (ASW) (Seasalts, Sigma, St. Louis, MO) for marine strains, or sterile physiological water (PW) (NaCl, 0.9%, wt/vol) for non-marine strains. Sterilization of suspension solutions was completed by filtration through 0.22 µm then 0.1 µm pore-size filters (Nuclepore, Costar, Pleasanton, CA).

Appropriate volumes of cellular suspensions were then dispersed in 1000 ml of sterile ASW or PW (acid-washed bottles rinsed with sterile water), to obtain initial abundance of 10^7 to 10^8 cells ml^{-1}. These microcosms were maintained at room temperature (ca 22 ± 2°C) on an orbital shaker (Rotatest 74403, Bioblock Scientific, Illkirch) at 150 rpm.

Samples for analyses were taken just after inoculation (T0) and after 1 (T1), 7 (T2), 14 (T3), and 28 d (T4) of incubation.

Particulate organic carbon (POC) determination: At each sampling time, 3 replicate samples (50 ml) were withdrawn and filtered in a clean acid-washed and rinsed glass filter unit (Millipore) through 2 stacked precombusted (450°C, ≥4 h) GF/F glass fiber filters (Whatman, 25 mm). Filters were dried at 60°C for >24 h and were assayed on a Leco CHN analyzer. At the beginning of the experiment, three 50 ml samples of suspension medium (ASW and PW) were filtered on 2 stacked precombusted GF/F filters to check for carbon contribution of suspension medium. At each time, 2 blank precombusted GF/F filters (no water filtered) were assayed. These control filters contained no measurable amounts of carbon. On the other hand, bacterial abundance (see hereafter) in each filtrate was estimated, showing that the 2 stacked filters retained at least 99% of bacterial cells in samples (data not shown).

Cell counts and biovolume determinations: Triplicate samples (2 ml) were taken at each sampling time and fixed with 0.2 µm filtered formaldehyde (final concentration 2%, vol/vol). After 1 h of fixation, samples were stained with DAPI (final concentration 2.5 µg ml^{-1}) for 24 h at 5°C in the dark. At the end of staining time, cell abundance was immediately measured by flow cytometry (ACR 1400SP, Bruker-Spectrospin, Wissembourg, France) according to Troussellier et al. (1993).

At the same time, a subsample was filtered onto a black Nuclepore filter (0.2 µm pore size) which was stored at 5°C in the dark, and examined, within 48 h, with an epifluorescence microscope (Olympus BH with a 100× UVFL iris objective) to determine size of DAPI stained bacteria. Photographs (Kodak Tmax ASA 400) were taken from 10 randomly selected fields, then projected onto a digitizer tablet (final magnification, ×5000). Dimensions of 50 cells per sample were measured with a computer-assisted digitizer. Cell volume (V) was calculated assuming that the shape of bacteria was spherical or cylindrical with hemispheric ends: $V = (\pi w^2/4)(l - w/3)$, where w is the width and l is the length of the cell (Bratbak 1985).

Calibration was performed with microphotographs of a slide micrometer (Zeiss) and microspheres (Polysciences, Warrington, PA) of 0.51 and 0.75 µm diameter.

Results. Changes in measured variables and computed conversion factors for marine and non-marine strains are reported in Figs. 1 & 2, respectively. Cell abundances showed either a slow but significant decreasing trend with time (all marine strains, *Aeromonas hydrophyla*, and *Enterobacter cloacae*), or stability (the other non-marine strains). In all incubations the average POC and cell volume values also showed a significant decrease with time. The ratio between cell length and width decreased with decreasing average cell volume, with the exception of *Vibrio fischeri* cells which have an initial low ratio (Table 1). Thus, cells tended to be more coccoid during incubation.

Carbon content per cell (C_c): There was no significant increase or decrease of carbon content per cell (C_c) with time, when the whole starvation period was taken into account (Figs. 1 & 2). However, during the first 24 h or the first 7 d, C_c values generally showed a limited decrease and remained stable thereafter.

The small coefficient of variation associated with mean C_c value of each strain confirms the fairly constant value of their cellular carbon density (Table 2). While significant differences among C_c values of the different strains were detected by a 1-way ANOVA (F-value = 9.687; $p < 0.0001$), there was only a factor of 2.3

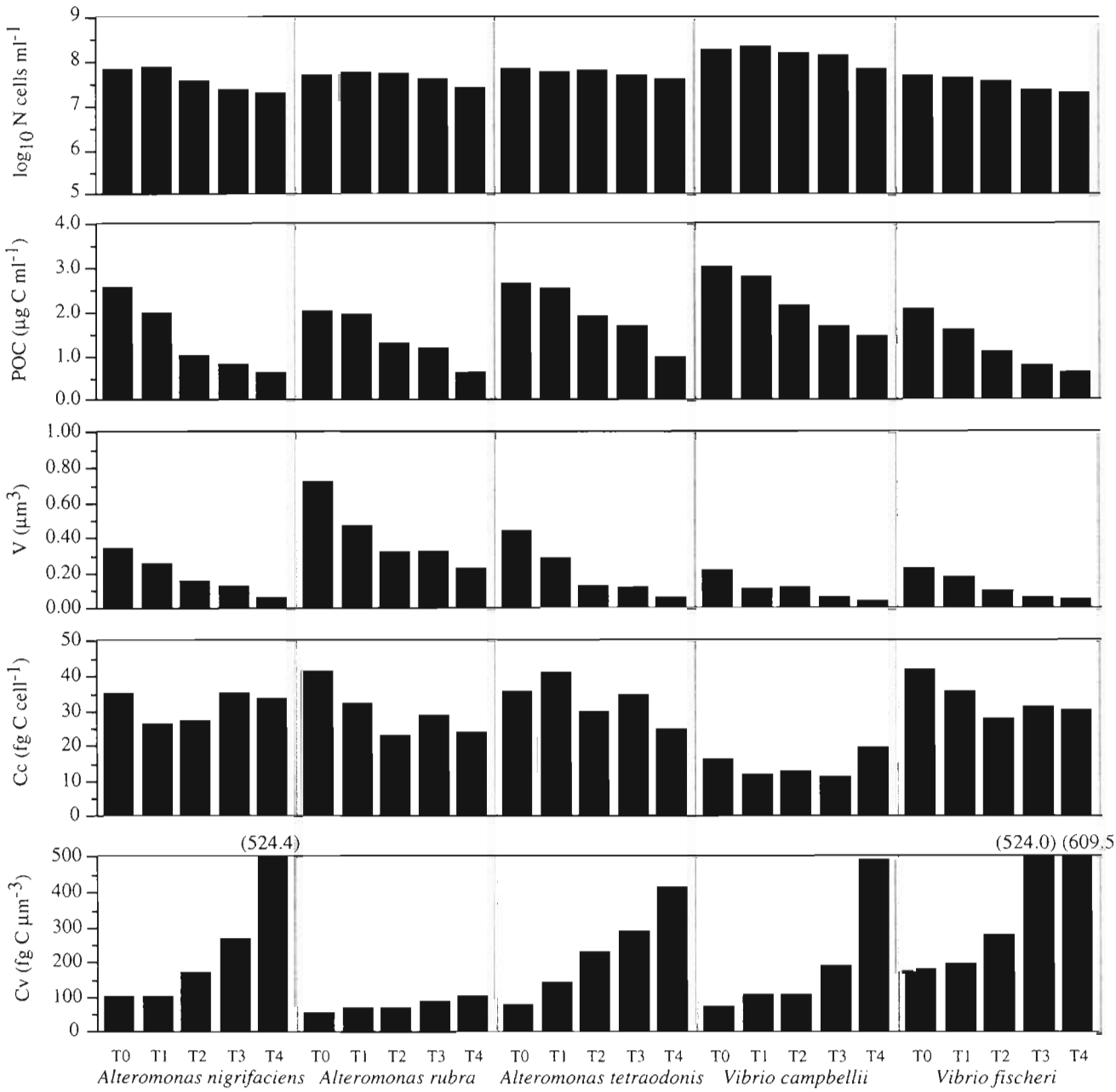


Fig. 1. Changes in cell abundances (N), particulate organic carbon (POC), cell volume (V), carbon content per cell (C_c), and carbon density (C_v) for marine strains during starvation. Data are mean of triplicate measurements

between the lowest (14.51, *Vibrio campbellii*) and the highest (33.45, *V. fischeri*) mean C_c values.

Carbon content per cellular volume unit (C_v): In contrast, the amount of carbon per cellular volume unit (C_v) exhibited a significant increase during incubation for each strain (Figs. 1 & 2).

The slopes of the linear regression model between C_v values and time showed that, except for *Alteromonas rubra*, marine strains have a higher C_v increase rate than non-marine strains (Table 3). At the end of the starvation period (28 d), there was a factor of 9 between the lowest (68.84, *Escherichia coli*) and the highest (609.52, *Vibrio fischeri*) C_v values.

C_c and C_v versus volume (V) relationships: When C_c values were plotted versus volume values, there was no significant relationship between the 2 variables (Fig. 3a), but plotting C_v versus volume values showed a strong non-linear relationship (Fig. 3b) which was fit-

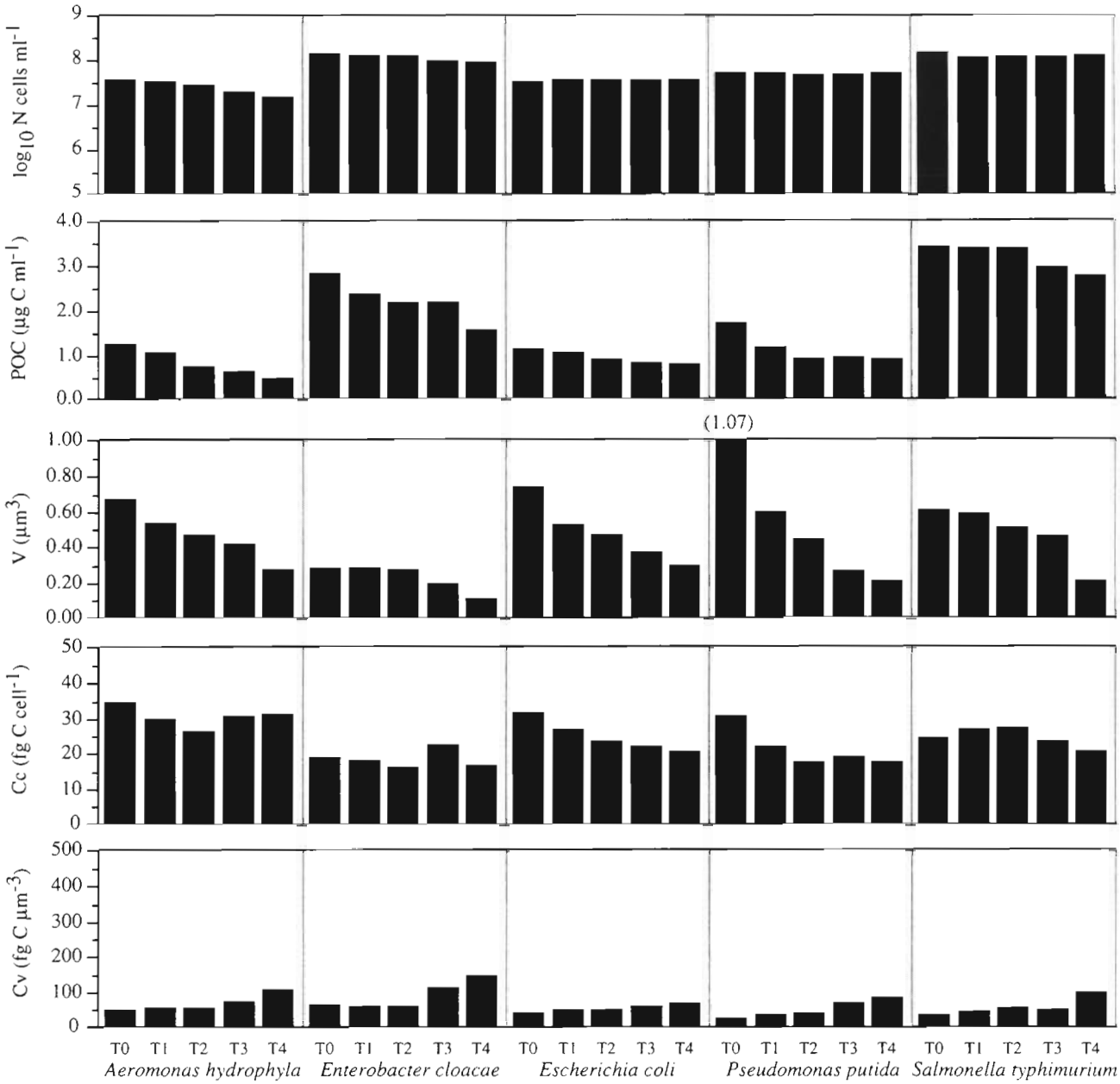


Fig. 2. Changes in cell abundances (N), particulate organic carbon (POC), cell volume (V), carbon content per cell (C_c), and carbon density (C_v) for non-marine strains during starvation. Data are mean of triplicate measurements

ted by the following power function ($C_v = \alpha V^{\beta}$):

$$C_v = 25.73\ V^{-0.981} \quad (r^2 = 0.847,\ p < 0.0001,\ n = 50)$$

The 95% confidence intervals (lower and upper) for α and β are: α, 21.54 and 30.22; β, −1.110 and −0.867.

This equation was obtained from the major axis regression method applied to log-transformed variables, because there was error associated with the measurement of both variables of the model (Sokal & Rohlf 1981) and because the data displayed a bivariate log-normal distribution. However, as underlined by Jackson & Somers (1991), the analysis of association between 2 variables x and z, where $z = y/x$, may result in an 'artificial' correlation because of the lack of independence between the ratio and its denominator. Thus, in this case, the null value, against which a sample correlation is to be tested, is not zero, but the correlation found after, and thus generated by, the transformation (Chayes 1971). As recommended by Jackson & Somers (1991), we used a randomization test (based

Table 1. Mean length (L) and width (W) (in µm; SD in parentheses) and shape factor ($F = L/W$) values of each strain at the beginning (T0) and at the end (T4) of starvation experiments

Bacterial strain	Time	Length	Width	F
Marine				
Alteromonas nigrifaciens	T0	1.13 (0.45)	0.70 (0.05)	1.614
	T4	0.52 (0.22)	0.36 (0.14)	1.445
Alteromonas rubra	T0	1.69 (0.46)	0.79 (0.05)	2.140
	T4	0.88 (0.27)	0.63 (0.13)	1.391
Alteromonas tetraodonis	T0	1.43 (0.59)	0.62 (0.16)	2.314
	T4	0.57 (0.16)	0.42 (0.10)	1.371
Vibrio campbellii	T0	0.99 (0.39)	0.58 (0.08)	1.701
	T4	0.49 (0.09)	0.38 (0.07)	1.280
Vibrio fischeri	T0	0.86 (0.23)	0.66 (0.12)	1.312
	T4	0.50 (0.12)	0.39 (0.10)	1.305
Non-marine				
Aeromonas hydrophyla	T0	1.77 (0.56)	0.73 (0.11)	2.440
	T4	0.99 (0.64)	0.59 (0.17)	1.680
Enterobacter cloacae	T0	1.20 (0.39)	0.58 (0.15)	2.050
	T4	0.70 (0.28)	0.45 (0.14)	1.564
Escherichia coli	T0	1.91 (0.71)	0.74 (0.07)	2.569
	T4	1.18 (0.39)	0.62 (0.08)	1.905
Pseudomonas putida	T0	1.87 (0.57)	0.91 (0.12)	2.067
	T4	0.85 (0.34)	0.61 (0.08)	1.155
Salmonella typhimurium	T0	1.43 (0.40)	0.79 (0.15)	1.804
	T4	0.86 (0.21)	0.65 (0.09)	1.341

on 1000 permutations) and found a probability $p \leq 0.001$ that the observed correlation was obtained by chance, and so we rejected the null hypothesis of random association.

When applied to marine or non-marine strains only, the parameters of the power function (lower and upper 95% confidence limits) were not significantly different: marine strains: $\alpha = 34.34$ (20.03, 44.88), $\beta = -0.918$ (−1.160, −0.722); non-marine strains: $\alpha = 29.55$ (25.28, 33.80), $\beta = -0.749$ (−0.914, −0.606).

Table 2. Mean, standard deviation (SD) and coefficient of variation (CV) of C_c (fg C cell^{-1}) values of each strain

Bacterial strain	Mean	SD	CV (%)
Marine			
Alteromonas nigrifaciens	31.63	4.20	13.3
Alteromonas rubra	29.96	7.50	25.0
Alteromonas tetraodonis	33.26	6.09	18.3
Vibrio campbellii	14.51	3.48	24.0
Vibrio fischeri	33.45	5.36	16.0
Non-marine			
Aeromonas hydrophyla	30.59	2.84	9.3
Enterobacter cloacae	18.57	2.44	13.2
Escherichia coli	24.96	4.55	18.2
Pseudomonas putida	21.42	5.54	25.9
Salmonella typhimurium	24.07	3.00	12.5

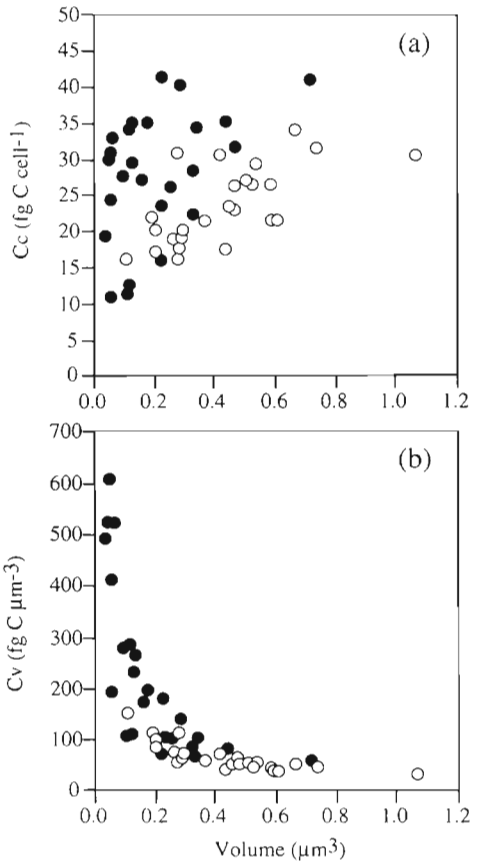


Fig. 3. Relationships between (a) carbon content per cell (C_c) and volume values and (b) between carbon density (C_v) and volume values. (○) Non-marine strains; (●) marine strains

Discussion. ***Carbon content per cell (C_c):*** Regarding the conversion factors among the 10 bacterial species, even if significant differences were shown, carbon content per cell (C_c) was less variable than carbon per

Table 3. Rate of C_v increase (d^{-1}; R^2 and p values in parentheses) as deduced from linear regression model between C_v values and duration (d) of starvation incubation

Bacterial strain	Rate
Marine	
Alteromonas nigrifaciens	15.14 (0.986, 0.0007)
Alteromonas rubra	1.57 (0.948, 0.0051)
Alteromonas tetraodonis	11.02 (0.954, 0.0043)
Vibrio campbellii	14.27 (0.919, 0.0101)
Vibrio fischeri	16.30 (0.914, 0.0110)
Non-marine	
Aeromonas hydrophyla	2.12 (0.957, 0.0038)
Enterobacter cloacae	3.29 (0.902, 0.0135)
Escherichia coli	0.82 (0.925, 0.0089)
Pseudomonas putida	1.90 (0.918, 0.0103)
Salmonella typhimurium	1.97 (0.888, 0.0164)

volume (C_v). In fact, a limited decrease of C_c values was observed during the first days of starvation. This could be the consequence of short-term degradation of some cellular constituents, such as RNA (Mason et al. 1986, Mason & Egli 1993, Cabral 1995). The degradation of other major constituents of the cellular carbon content (e.g. protein) is known to show a small decrease during midterm starvation due to the decrease of endogenous metabolism (Mason et al. 1986, Moyer & Morita 1989).

C_c values showed a relatively low range of variation (26.24 ± 1.08 fg C cell^{-1}, mean ± SE). This value is close to the cell carbon content reported by Lee & Fuhrman (1987) (20 ± 0.8 fg C cell^{-1}) and Tuomi et al. (1995) (15 to 42 fg C cell^{-1}), but lower than average per cell carbon content estimated by Bratbak (1985) (106 to 214 fg C cell^{-1}), Bjørnsen (1986) (64 fg C cell^{-1}) or Kroer (1994) (112.9 ± 68.9 fg C cell^{-1}).

Contribution of measurement errors to the variation of C_c obtained in this study is limited, because the mean coefficient of variation associated with replicate measurements of cell counts or POC was low (6.1 and 15.7%, respectively).

Another more probable reason for the C_c variations may be the different experimental conditions for C_c estimations. Most of the data was obtained from growth experiments conducted on filtered natural waters that may be enriched by organic substrates (see Nagata & Watanabe 1990). As a consequence, measurements were done on cell populations in growth or stationary phase. During these phases, dry weight, protein and nucleic acids content per cell increase (Bremer & Dennis 1987). Thus, the large differences in C_c values reported in the literature may be, at least partly, the result of differences in growth rate depending on nutritional conditions.

Carbon content per volume (C_v): Clearly, there is a progressive increase in carbon cellular 'concentration' as starvation takes place, but increase rates were very different among species: it was minimal for *Escherichia coli* (0.82 fg C µm^{-3} d^{-1}) and maximal for *Vibrio fischeri* (16.3 fg C µm^{-3} d^{-1}). In general, marine strains showed higher C_v increase rates than non-marine strains. As a consequence, at the end of the experiment mean values (±SE) obtained for marine strains were higher (218.49 ± 33.32 fg C µm ^{3}) than for non-marine strains (64.60 ± 5.60 fg C µm^{-3}). This is consistent with the observations of Nagata & Watanabe (1990).

The carbon to volume ratio is directly related to the water content of bacteria which in turn depends on the osmotic conditions in the cell (Fagerbakke et al. 1996). If we assume, following Bjørnsen (1986), a partial dry matter density of 1.6 g cm^{-3} and a 50% carbon/dry weight ratio, C_v values higher than 500 fg C µm^{-3} would imply very high dry matter content (>60%) or low water content (<40%). Such high C_v values have been observed only for marine strains at the end of starvation periods. From the power-function equation, it may be shown that values higher than 500 fg C µm^{-3} would be obtained for cellular volumes lower than 0.05 µm^3. These low values were only reached by marine strains (min. 0.04 µm^3), while lowest values for non-marine strains were around 0.1 µm^3. Simon & Azam (1989) have already reported that marine diminutive bacteria are very 'dry'.

So, we can hypothesize that the highest mean values and increase rate of C_v for marine strains starved in ASW are the consequence of a faster decrease of their water content than for non-marine strains starved in PW.

As suggested by different authors, the variability of C_v values in the literature is probably caused by the use of different criteria to estimate the size of bacterial cells. This effect was supposed to be reduced in our study, because cell dimensions were measured in the same way. We obtained no significant relationship between the coefficient of variation and mean values of cell volume. Therefore, most of the variation in our data may be attributed to species and starvation effects.

C_c and C_v versus volume relationships: The increase of C_v was significantly correlated with the decrease of mean cell volume (Fig. 3b). This relationship was observed for each strain during the incubation period (data not shown). On the whole, a power function was used to fit the data because of the non-linear form of the relationship, as already reported by Lee & Fuhrman (1987). This model indicates that C_v of the cells increases greatly as the cellular volume decreases. The parameters of the equations were not significantly different for marine and non-marine strains. This is consistent with the non-significant differences between cell volume:protein relationships for freshwater and marine natural bacterial assemblages reported by Simon & Azam (1989). However, in spite of careful statistical analysis (see 'Results' section), the fact remains that there is no biological interpretation of the parameters of these equations (Norland 1993).

Despite the relatively small number of bacterial species used in this study, our results support the hypothesis that C_v and, to a lesser extent, C_c values are species dependent. C_v is also greatly affected by starvation conditions. However, it seems that the differences in C_v between marine and non-marine bacteria observed in this study and by others (Nagata & Watanabe 1990) are not the consequence of different relationships between C_v and volume but, more likely, of lower cell volume and its faster decrease for marine bacteria than for non-marine bacteria. The cell shrinkage process may be a general adaptative process to

starvation or sharp nutrient limitation, which may be more frequently experienced by marine than by non-marine bacteria. An increase in the surface/volume ratio of cells, which is achieved by reduction of cell size, may facilitate the uptake of scarce substrates (Gottschal 1992). Size reduction may also lead to minimization of maintenance requirements (Oliver 1993). The mechanism whereby marine bacteria, starved in artificial seawater, reduce their water content faster than do non-marine bacteria in physiological water remains unclear and merits further examination.

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