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Content of carbon, nitrogen, oxygen, sulfur and phosphorus in native aquatic and cultured bacteria

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ABSTRACT: The content of carbon, nitrogen, oxygen, phosphorus and sulfur was measured in individual cells from 6 native aquatic samples and 4 samples of cultured bacteria by X-ray microanalysis using a transmission electron microscope (TEM). The molar C:N:P ratio for the pooled sample was 50:10:1. From length and width measurements of unfixed air-dried cells we estimated cell volumes over a total range of 0.0026 to 15.8 μm^3, and mean C:volume ratios of 30 to 162 fg μm^{-3} for the samples included. For the marine samples we found mean N:C ratios of 0.25 to 0.28, while cells from fresh or brackish waters had mean N:C ratios of 0.17 to 0.20, indicating differences in nutrient availability. The P:C ratios for the samples analyzed varied from 0.040 to 0.090, with a pooled mean of 0.052, which is approximately twice that of the Redfield ratio for P:C. For O:C ratios we estimated a pooled mean of 0.37 and a range of 0.22 to 0.77 for all samples. We may conclude that slow-growing or non-growing cells have low O:C ratios. The mean S:C ratio for all samples was 0.031, with a range of 0.016 to 0.084 for the sample means. A general conclusion is that single-cell analyses of elemental composition give important information on the physiological conditions of cells and on possible nutrient limitations. The rationale for this is the assumption that changes in macromolecular composition are due to nutrient availability.

KEY WORDS: X-ray microanalysis · Carbon Nitrogen · Oxygen · Sulfur · Phosphorus · Volume · Ratios

INTRODUCTION

Bacteria are important participants in the turnover of organic and inorganic material and are also important for production in aquatic environments. In lakes this secondary production may, in terms of carbon, equal primary production (Morris & Lewis 1992). It has been suggested that the flux of organic C from primary production strongly regulates bacterial production (Cole et al. 1988), but inorganic nutrients like P and N may also contribute to bacterial growth regulation.

Bacterial biomass is commonly estimated through measurements of total cell numbers, bacterial biovolume, and the use of conversion factors for C:volume ratios (Fry 1990 and references therein). C:volume ratios over a broad range have been reported for native aquatic bacteria (Watson et al. 1977, Bratbak 1985, Kogure & Koike 1987, Lee & Fuhrman 1987). Furthermore, the N:C and P:C ratios based on bulk measurements reported for bacteria also show large variations (Bratbak 1985, Vadstein & Olsen 1989, Tezuka 1990). Despite the variation, the relative amount of nitrogen and particularly of phosphorus is higher in bacteria than in algae. Goldman et al. (1987) found a molar C:N:P ratio of 45:9:1 for bacteria, whereas the commonly referred to molar Redfield ratio for algae is 106:16:1 (Redfield et al. 1963).

Despite their importance for an understanding of the microbial ecosystem, relatively few studies of the elemental composition of bacteria and of how this composition is influenced by growth conditions have been presented. The elemental composition of bacteria determines the quality of the material that is transported to higher trophic levels by grazing, and it also controls their role in the regeneration process, whether or not they excrete or consume mineral nutrients (Parnas 1975, Goldman et al. 1987, Goldman & Dennet 1991). The elemental composition of bacteria also reflects the conditions under which they grow and may point to possible limiting factors (Egli 1991). Several studies have shown that the growth rate of microor-

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ganisms is more closely related to the cellular content of a limiting nutrient than to external nutrient concentrations (Droop 1983, Vadstein et al. 1988)

A large discrepancy between total counts and viable counts of bacteria from aquatic environments may indicate that a considerable fraction of the cells are non-growing, dormant or inactive (Hobbie et al. 1977, Fry & Zia 1982, Zweifel & Hagström 1995). If this non-growing or dormant fraction is nutrient limited, measurements of the elemental composition should reveal the limitation to the extent that it is possible to measure structural elements like carbon, nitrogen, and phosphorus at single-cell levels.

Most microbiological methods are based on bulk techniques, which determine a population mean for a measured property. However, with the advent of single-cell techniques, e.g. flow cytometry and quantitative image analysis, the importance of the heterogeneity of cultures has been appreciated, a heterogeneity which goes beyond that caused by binary fission alone. Kell et al. (1991) have shown that cultures of *Micrococcus luteus* may have a bimodal distribution of autofluorescence. Transmission electron microscopes (TEM) equipped with energy dispersive X-ray detectors have been used for quantitative measurements of the elemental composition of single cells (Heldal et al. 1985, Fagerbakke et al. 1991, Norland et al. 1995).

In this study we use a method developed by the authors (Norland et al. 1995) based on X-ray microanalysis (XRMA) in TEM to measure the major structural elements (carbon, nitrogen, oxygen, phosphorus and sulfur) in single bacteria from both cultured and native samples. The objective is to describe the variation of elemental composition within and among samples and to study how nutritional status and growth conditions are reflected in the elemental composition of the cells.

MATERIAL AND METHODS

Native bacteria were sampled from 4 locations on 6 occasions: Raunefjorden, 15 km south of Bergen, Norway (60° 16' N, 5° 14' E) in June and October 1993; Lake Kalandsvatnet, 10 km south of Bergen (60° 17' N, 5° 25' E) in October 1992; Tvärminne, Finland (59° 51' N, 23° 16' E) in July 1993; Knebel Vig, Aarhus Bight, Denmark (56° 10' N, 10° 30' E) in June 1992 and July 1994.

Samples of laboratory-grown bacteria *Escherichia coli* and *Vibrio natriegens* were obtained from both growing cultures and from cultures in the stationary phase. The cultures and samples were prepared as follows:

Escherichia coli B6 wildtype was grown in a medium with a low potassium concentration. NaH_2PO_4 (4.5 mM), Na_2HPO_4 (1 mM), $NaNO_3$ (10 mM), KCl (1 mM), $MgSO_4$ (10 mM). The cells were grown in a 100 ml batch culture at 37°C on a rotary shaker. After 24 h, in the stationary growth phase, samples for XRMA and CHN (carbon, hydrogen, nitrogen) analysis were taken. The culture was then diluted by adding 2 parts (70 ml) 1% Nutrient Broth (Oxoid) to 30 ml of the culture. Samples from exponentially growing bacteria were taken after 1 h of incubation.

Vibrio natriegens NCMB857 was grown in BHI (brain heart infusion) medium (Oxoid) (37 g l^{-1} BHI was dissolved in 70% artificial sea water and sterilized). The cells were grown at 35°C in a water bath with shaking. Samples were taken in the mid-exponential growth phase (1 h) and 30 min after the onset of the stationary phase, i.e. 2 h after the start of incubation. Growth was measured as light absorption at 620 nm using a spectrophotometer

The bacteria were harvested by centrifugation onto aluminium grids (100 mesh; Agar Scientific, Stansted, Essex, UK) supported with carbon-coated formvar film. Neither fixatives nor stain were applied. The cells were air-dried at ambient conditions and analyzed in a JEOL 100 CX transmission electron microscope operated in scanning mode and equipped with a Tracor Z-MAX 30 detector featuring silicon crystal and Norvar single window for light-element detection. The microscope was operated at 80 kV, with a magnification between 10000× and 60000×, and the grids were placed between high-purity carbon plates at a 38° nominal take-off angle. X-ray spectra were accumulated for 50 s (live time) in a Tracor multichannel analyzer and stored on floppy disks. The scanned area, bacterial length and width were measured on the CRT (cathode ray tube) screen, which was calibrated with latex beads (Agar Scientific). A particle-free area near each bacterium was chosen for the film background spectrum.

Processing of spectra. The spectra were transformed to MS-DOS-compatible binary files using a laboratory computer (MINC, Digital Equipment Corporation). All software and calibration procedures involved were developed by the authors, and the principles involved are described by Norland et al. (1995). Volume was determined by assuming the cell to be cylindrical with hemispherical ends. Dry matter was determined as the sum of all measured elements, assuming a hydrogen content equal to 1/6 of the carbon content.

Due to the wide range of cell sizes included in the analyses and to the skewed (approximately log-normal) distribution of the measured properties of the cells, we chose to calculate all means and standard deviations from log-transformed data. These were

then converted back to the linear domain (antilogs of log-transformed data) for presentation. This implies that all means are geometric, which for this set of data are 10 to 30% lower than the arithmetic ones. We report the standard deviation of log-transformed data as arithmetic coefficients of variation (CV), an approximation that holds for small values.

For the ratio of 2 parameters, z and y, $x_i = z_i/y_i$. The weighted among-group coefficient of variation (CV_A) was computed using:

$$CV_A = \sqrt{\frac{\sum_g \{n_j[\ln(\bar{x}_j) - \ln(\hat{x})]^2\}}{\sum_g n_j - 1}} \quad (1)$$

where $\hat{x}$ is the grand mean of the pooled population, and $\bar{x}_j$ is group mean and n_j is the sample size of the group. The weighted within-group coefficient of variation (CV_W) was calculated using:

$$CV_W = \sqrt{\frac{\sum_g (n_j CV_j^2)}{\sum_g n_j - 1}} \quad (2)$$

where CV_j is the coefficient of variation of the group. The total relative variance is the squared sum of CV_A and CV_W. In this context we used these formulae to compute CV_A and CV_W using the 10 samples as groups.

The linear regressions between variables (C vs volume, and N, O, P and S vs C) were based on logarithmic transformations, using a type II model (Norland et al. 1987):

$$\ln(y) = b\ln(x) + a \quad (3)$$

where x and y are the 2 variables, a is the value of y when $\ln(x) = 0$ (i.e. $x = 1$) and b is the scale coefficient and is equal to unity when the 2 variables are proportional.

RESULTS

Cellular values of volume, dry matter, and the elements carbon, nitrogen, oxygen, phosphorus and sulfur were measured in 404 single bacteria from 10 different sources, and the sample means are shown in Table 1. Native cells were sampled from 4 different locations covering lake water, brackish water and sea water, and from some of these locations during different seasons, and from cultures of *Escherichia coli* and *Vibrio natriegens* at different growth stages. The estimated mean volume of the cells varied over nearly 2 orders of magnitude, from 0.11 to 3.8 μm^3, while the total range of volumes covered more than 3 orders of magnitude: 0.0026 to 15.8 μm^3. The laboratory-grown bacteria were the largest. They showed significantly different volumes between the growing and stationary phases. The total range of the per cell dry weight of elements was more than 3 orders of magnitude, from 1.4 fg to 1.9 pg.

A plot of carbon versus volume single cells values is shown in Fig. 1. The sample means for C:volume ratios varied from 32 to 160 fg C μm^{-3} (Table 2); within the total pooled sample the ratio varied by a factor of 30, from 14 to 400 fg C μm^{-3} (Fig. 1). The pooled regres-

Table 1 Volumes and element content of bacteria from different locations and cultures. Geometric means of single cell measurements with standard error. n: number of cells analyzed

	Volume (μm^3)	Dry matter (fg $cell^{-1}$)	Element content (fg) C	N	O	P	S	n
Location, date								
Raunefjorden, June 1993	0.11 ± 0.01	21 ± 2	9 ± 1	2.2 ± 0.3	4.0 ± 0.4	0.50 ± 0.05	0.43 ± 0.05	62
Raunefjorden, October 1993	0.28 ± 0.08	37 ± 8	19 ± 4	5 ± 1	5 ± 1	0.8 ± 0.2	0.35 ± 0.09	20
Knebel Vig, Denmark, 1994	0.20 ± 0.02	26 ± 2	12 ± 1	2.6 ± 0.2	4.7 ± 0.4	0.47 ± 0.04	0.44 ± 0.05	95
Knebel Vig, 1992	0.21 ± 0.03	22 ± 3	7 ± 1	1.6 ± 0.2	5.1 ± 0.7	0.46 ± 0.07	0.56 ± 0.07	55
Tvärminne, Finland	0.31 ± 0.09	60 ± 20	31 ± 10	5 ± 2	9 ± 3	1.4 ± 0.3	0.6 ± 0.2	17
Lake Kalandsvatnet, Norway	0.41 ± 0.03	39 ± 3	21 ± 2	4.4 ± 0.3	4.9 ± 0.4	1.05 ± 0.08	0.30 ± 0.02	87
Species								
Vibrio natriegens								
Growing	3.5 ± 0.5	850 ± 90	350 ± 40	90 ± 10	120 ± 10	17 ± 2	13 ± 1	11
Stationary	0.93 ± 0.06	145 ± 5	62 ± 4	16 ± 1.0	24 ± 1	3.4 ± 0.1	2.5 ± 0.1	11
Escherichia coli								
Growing	3.8 ± 0.4	710 ± 80	350 ± 40	100 ± 10	120 ± 20	31 ± 4	9 ± 1	26
Stationary	0.7 ± 0.1	180 ± 20	110 ± 10	24 ± 3	24 ± 3	6.7 ± 0.7	1.7 ± 0.2	20

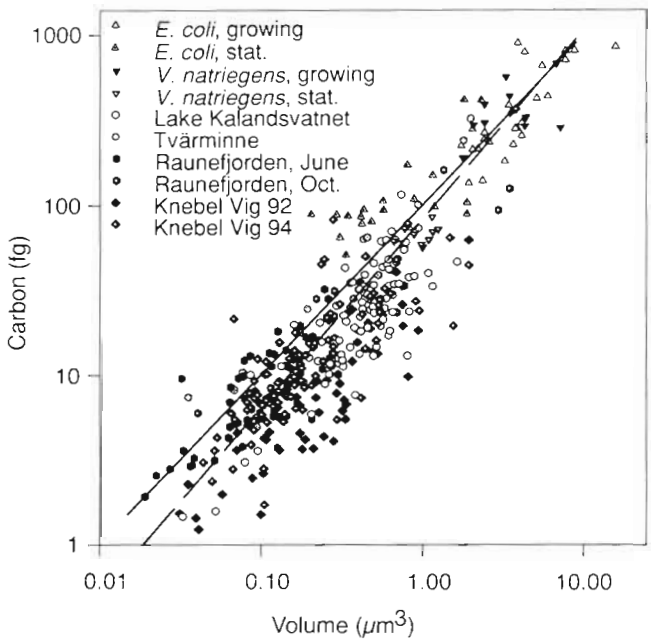

Fig. 1. Log-log plot of carbon content versus cell volumes for all cells analysed. Solid line: 100 fg C μm^{-3}; dashed line: regression line

sion of carbon versus volume is expressed in Eq. (4) (Table 3). The regression line (dashed line) from Eq. (4) is shown in Fig. 1 together with the 100 fg C μm^{-3} line (solid line).

Per cell amounts of nitrogen, oxygen, phosphorus and sulfur content versus carbon are shown in Fig. 2. For all these relationships the regressions lines are drawn from Eqs. (5) to (8), Table 3. In addition, the Redfield ratios for N:C and P:C are drawn (dashed lines). The mean pooled N:C ratio is approximately 30% higher than the Redfield ratio, while the pooled P:C ratio is nearly 3 times higher than the Redfield ratio. The mean pooled values of O:C and S:C are 0.37 and 0.031, respectively.

Elemental ratios of bacteria from the different locations are presented in Table 2. The sample means for the N:C ratio had values in the range of 0.17 to 0.29. The marine bacteria had means from 0.22 to 0.26 while the brackish water and freshwater had means from 0.17 to 0.22. The highest N:C value, 0.29, was found for growing *Escherichia coli*. The highest mean P:C ratio was found for growing *E. coli* and in the bacteria sampled from Knebel Vig in 1992, whilst only small varia-

Table 2. C:volume ratio and ratios of nitrogen, phosphorus, and oxygen relative to carbon (wt:wt) of the samples in Table 1. Values are given with standard error

	C:volume ratio (fg C μm^{-3})	Weight ratio			
		N:C	P:C	O:C	S:C
Location, date					
Raunefjorden, June	78 ± 4	0.26 ± 0.01	0.057 ± 0.004	0.46 ± 0.02	0.049 ± 0.003
Raunefjorden, October	67 ± 7	0.24 ± 0.01	0.041 ± 0.005	0.26 ± 0.02	0.019 ± 0.003
Knebel Vig, 1994	60 ± 3	0.22 ± 0.01	0.040 ± 0.003	0.40 ± 0.02	0.037 ± 0.003
Knebel Vig, 1992	32 ± 2	0.24 ± 0.01	0.069 ± 0.005	0.77 ± 0.05	0.084 ± 0.008
Tvärminne	103 ± 9	0.17 ± 0.02	0.043 ± 0.007	0.29 ± 0.03	0.019 ± 0.003
Lake Kalandsvatnet	53 ± 2	0.20 ± 0.01	0.049 ± 0.003	0.23 ± 0.01	0.016 ± 0.001
Species					
Vibrio natriegens					
Growing	100 ± 10	0.26 ± 0.01	0.050 ± 0.003	0.34 ± 0.01	0.035 ± 0.002
Stationary	67 ± 5	0.26 ± 0.03	0.055 ± 0.004	0.39 ± 0.03	0.040 ± 0.003
Escherichia coli					
Growing	92 ± 6	0.29 ± 0.01	0.090 ± 0.005	0.35 ± 0.02	0.026 ± 0.003
Stationary	160 ± 20	0.22 ± 0.01	0.062 ± 0.002	0.22 ± 0.01	0.016 ± 0.001

Table 3. Regression equations between combinations of variables and the contribution to the total variation of their ratios from within-group variation and among-group variation. Volume (Vl) has the unit μm^3, all elements given in fg

Relation	Regression equation	Coefficient of variation		Eq. no.
		Within (CV_W)	Among (CV_A)	
C vs Volume	$\ln(C) = (1.12 \pm 0.03) \times \ln(Vl) + (4.28 \pm 0.04)$	0.46	0.38	(4)
N vs C	$\ln(N) = (1.02 \pm 0.02) \times \ln(C) - (1.54 \pm 0.05)$	0.42	0.12	(5)
P vs C	$\ln(P) = (1.05 \pm 0.02) \times \ln(C) - (3.10 \pm 0.07)$	0.53	0.24	(6)
O vs C	$\ln(O) = (0.94 \pm 0.03) \times \ln(C) - (0.8 \pm 0.1)$	0.37	0.39	(7)
S vs C	$\ln(S) = (1.00 \pm 0.02) \times \ln(C) - (3.5 \pm 0.1)$	0.60	0.61	(8)

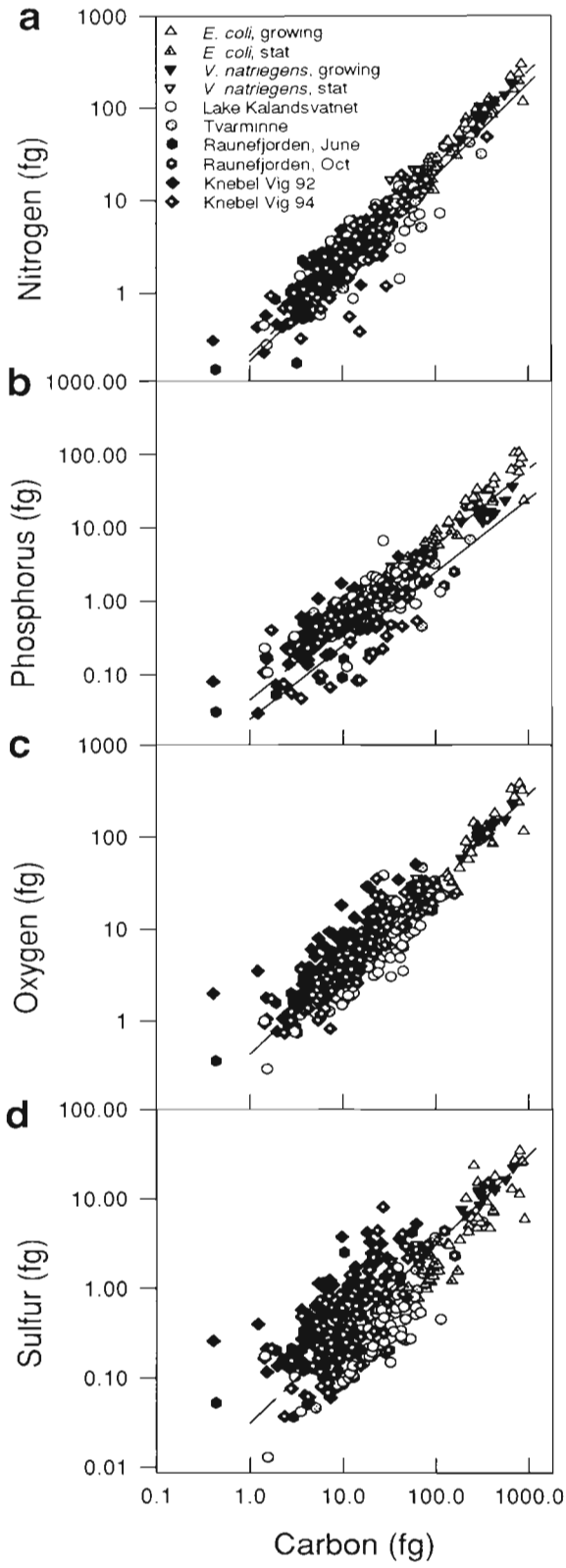

Fig. 2. Log-log plot of nitrogen, phosphorus, oxygen and sulfur versus carbon for all cells analysed. Solid lines: Redfield ratios (nitrogen and phosphorus); dashed lines: regression lines

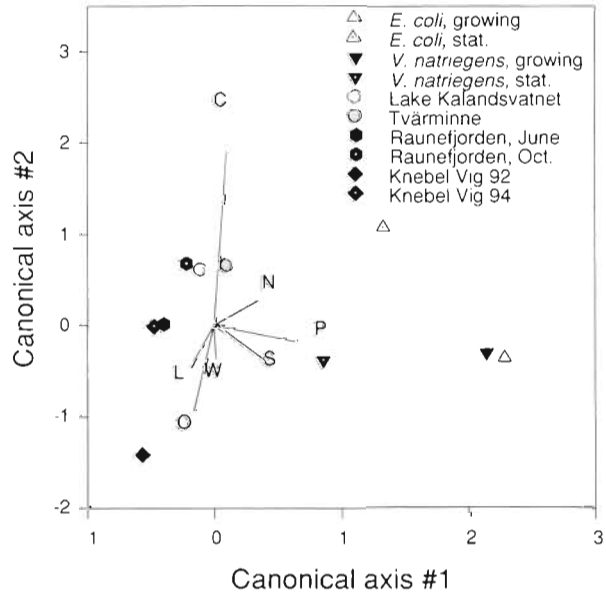

Fig. 3. Canonical variate plot of the first 2 canonical axes (first and second axes explain 45 and 30% of the total dispersion, respectively). L: bacterial length; W: bacterial width; C, N, O, P and S are the elements included

tions were determined among the other means. The pooled regressions between nitrogen, phosphorus, oxygen and sulfur versus carbon are given by Eqs. (2) to (5), Table 3, together with corresponding coefficients of variations within and among groups.

Fig. 3 shows a bivariate plot of the first 2 axes from canonical variate analysis using the 10 samples as groups. The 2 axes shown represent 45% (Axis 1) and 30% (Axis 2) of the total dispersion. The lengths and directions of the original axes indicate the contribution from each parameter to the dispersion of the cells. For Axis 1 P, S and N versus cell length (L), and O are the main contributors, while the contrast between C and N versus O, L, S and cell width (W) contribute most to the dispersion of Axis 2.

DISCUSSION

Cell volume

The mean cell volumes for bacteria from the native samples varied from 0.11 to 0.41 μm^3. Laboratory-grown *Escherichia coli* and *Vibrio natriegens* had mean cell volumes of 3.8 and 3.5 μm^3 during growth and 0.7 and 0.4 μm^3 during the stationary phase, respectively. We estimated cell volumes from measurements of lengths and widths at high magnification (10000× to 60000× enlargements) on air-dried but unfixed and unstained cells. In TEM the contrast is

usually sufficient to define edges for unstained bacteria, but the edges may be blurred at the highest magnifications The linear dimensions are generally determined with a precision better than 5%, giving an error less than 15% on volume. From comparative studies where lengths and widths of *E. coli* were measured on living cells (on a wet agar surface, light microscopy) and on air-dried cells (using TEM), we observed that, due to shrinkage, air-dried cells had a volume 10% less than the living cells (authors' unpubl. results). A more marked shrinkage, about a 40% reduction of volume, was observed for cells fixed (2% formaldehyde or 1.5% glutaraldehyde) before air-drying. During drying the cells collapse towards the formvar film due to evaporation of water, which has a high surface tension. Measurements of heights and widths of bacteria using an atomic force microscope (AFM) (Nano Scope III, Digital Instruments, Breda, The Netherlands), showed that air-dried cells from Raunefjorden (June 1993), harvested by centrifugation onto mica sheets, had a mean height:width ratio of 0.18 ± 0.06. The cross-section profiles of the cells were nearly rectangular, and apparently the cells collapsed towards the surface without a notable change in their width (G. Bratbak & M. Heldal unpubl. results).

Cell volumes were calculated assuming a model consisting of a cylinder with hemispherical ends (Heldal et al. 1985, Nagata 1986). Volume estimates will depend on the model chosen, e.g. assuming an ellipsoidal shape will give a 29% lower volume at a width:length ratio of 2. More emphasis should be placed on volume estimates and the models should be made more adaptive so that the volume of a wider range of the bacterial shapes actually found in nature can be better determined. In this context, image analysis should prove to be an important tool.

Dry matter

In this study the dry weight of cells was calculated as the sum of all measurable elements plus an additional contribution from hydrogen assumed to be 1/6 (wt:wt) of carbon (Norland et al. 1995). As shown in Table 1, the total range of mean dry weights for all samples was between 21 and 850 fg cell^{-1}. In the growing cultures, the per cell dry matter content was 1 order of magnitude higher than the dry matter content in native bacteria. The lower part of the size range was made up by very small bacteria. A subpopulation of the 9 smallest bacteria, in terms of dry weight, was treated separately. This subpopulation, which was comprised of bacteria from 4 different samples, had a mean dry weight of 4 fg, 1.5 fg carbon and a mean volume of 0.04 µm^3 Their mean N:C and P:C ratios were not significantly different from the means of the pooled sample (0.28 and 0.09, respectively). If we assume that all phosphorus in each of these cells (0.1 fg) was associated with DNA, an amount of 1.2 fg DNA cell^{-1} may be estimated. Most of the phosphorus is bound to the nucleic acid fraction (DNA and RNA). RNA:DNA ratios of cells will vary with growth rates and have been reported to be in the range of 1 to 10 (Skjold et al. 1973, Kato 1994). Therefore, a more realistic estimate for the DNA content would be less than 0.6 fg (genome size of <1000 kb). The weight of 1 *Escherichia coli* genome (9200 kb, or ~0.5 fg P) is 6 fg. Genome sizes of 1.5 to 2 fg (~0.15 fg P) for native soil bacteria have been reported by Bakken & Olsen (1991). Similar sizes of bacteria have been reported previously by Psenner & Sommaruga (1992). Schut et al. (1993) isolated an ultramicrobacterium with a cell volume of 0.06 µm^3 and a DNA content of 1 to 1.5 fg (~0.1 fg P), but in contrast to Moyer & Morita (1989) it was concluded that these small cells were actively growing.

The C:dry matter ratio of native marine bacteria was generally lower than that for native freshwater bacteria due to a higher content of inorganic ions (Mg^{2+}, Cl^-, Na^+, K^+) in the marine bacteria (authors' unpubl. results). We found mean C:dry matter ratios in the range of 30 to 60% for the various samples; however, C:dry weight ratios as low as 10% have been reported (Fry 1990).

Carbon-to-volume ratio

Mean C:volume ratios for the samples ranged from 32 to 160 fg µm^{-3} (Table 2). The lowest of these values is 1 order of magnitude lower than the values commonly used for conversion between volume and carbon (Table 4). The mean C:volume ratio for the pooled set of data (Fig 1) was estimated to be 63 fg C µm^{-3} which is also a value at the lower end of the C:volume ratios given in the literature (Table 4; Fry 1990). For the cultured bacteria, the C:volume values (Table 2) were in the same range as those determined earlier by use of the X-ray TEM method (Heldal et al. 1985, Nissen et al. 1987, Norland et al. 1987, 1995), and values determined by Watson et al. (1977).

The highest C:volume ratio in native bacteria, 103 fg C µm^{-3}, was found in the sample from Tvärminne. Growth conditions may influence the C:volume ratio, as seen for cells in the growing and stationary phases of *Escherichia coli* and *Vibrio natriegens*. For *E. coli*, the C:volume ratio for stationary phase cells (160 fg C µm^{-3}) was nearly twice that of growing cells (92 fg C µm^{-3}). The reverse situation was found for *V. natriegens* with 67 fg C µm^{-3} and 100 fg C µm^{-3} for stationary phase and growing cells, respectively.

The low C:volume ratio of the sample from Knebel Vig (1992) may be explained by the relatively high content of inorganic ions in these cells (25% wt:wt; data not shown).

If we assume that the dry matter of bacteria living in water has a specific density of 1.2 pg μm^{-3} and that carbon makes up 40% of this dry matter (Table 1), we may calculate a water content of 84% for the sample from Raunefjorden in June which has a C:volume ratio of 78 fg C μm^{-3} (Table 2). This is a value close to the shrinkage seen in AFM for the same sample (see 'Discussion: Cell volume' above). Most of the interest in the relation between carbon and volume stems from the need for a factor to convert biovolume into carbon. In fact, volume-to-carbon conversion enters the calculations in most of the work done involving microbial pools and rates. From the variation among means in C:volume ratio shown in Table 2 it is obvious that a 'universal' conversion factor cannot be used without the risk of introducing an error into one's estimates. The C:volume ratio is directly related to the water content of the bacteria, which in turn depends on the osmotic conditions in the cell. Thus, we think it is reasonable to assume that the C:volume ratio may vary over a fairly wide range.

The pooled set of data for carbon versus volume is presented in Fig. 1; the dashed line shows the regression line, while the solid line is drawn at 100 fg C μm^{-3}. The scale coefficient of the regression equation (Eq. 4, Table 3) is significantly higher than 1, which means that the C:volume ratio is lower for the small bacteria than for the larger ones. This result apparently contradicts previous related studies (Lee & Fuhrman 1987, Norland et al. 1987, Psenner 1990). It should, however, be noted that the among-sample variation for C:volume ratios is high (CV = 0.38; Table 3), and the number of samples is low. The CV for the pooled sample was 59%.

Nitrogen content

The mean values for nitrogen content in native bacteria were found to range from 1.6 to 5 fg $cell^{-1}$ (Table 1). The pooled mean N:C value is 0.23 (molar C:N = 5:1), which is consistent with values reported previously (Nagata 1986, Goldman et al. 1987). As seen from Table 2, the marine bacteria had similar N:C ratios, around 0.24, which is close to the value of 0.27 reported by Lee & Fuhrman (1987). However, N:C values of bacteria from marine environments have been reported to vary considerably and within a range from 0.07 to 0.35 (see Table 4 for further references). Assemblages of marine bacteria, grown in batch cultures on

Table 4. C:volume ratios and relative content of nitrogen, phosphorus, and sulfur (wt:wt) in bacteria. Data from the literature. M: marine; B: estuarine/brackish; F: freshwater; S: soil; f: formaldehyde; g: glutaraldehyde

Bacteria analyzed	Habitat	Fixative	fg C μm^{-3}	N:C	P:C	S:C	Source
Native	B, F	1% f	350				Bjørnsen (1986)
Native	F	2% f	154				Scavia & Laird (1987)
Escherichia coli	F	0.1% g	126–132				Watson et al. (1977)
P. putida, E. coli, B. subtilis	M, F	No	160–290				Bratbak & Dundas (1984)
Enriched native	M	No	133–400				Simon & Azam (1989)
Native	B, M	2% f	210–1610	0.11–0.41			Kroer (1994)
Enriched native and native	M	2% f	83–372	0.08–0.29			Kogure & Koike (1987)
Alkaligens, Pseudomonas	M	No	155–292	0.17–0.35			Kogure & Koike (1987)
Native	F	No	39–188	0.14–0.30			Nagata (1986)
Native	M	5% f	280	0.27			Lee & Fuhrman (1987)
Enriched native	F	1% f	150–170	0.11–0.21	0.017–0.040		Vadstein & Olsen (1989)
Enriched native and *P. putida*	B	5% g	560	0.15–0.22	0.005–0.16		Bratbak (1985)
A. globiformis and *E. aerogenes*	S	No	210–580	0.19–0.30	0.024–0.056		van Veen & Paul (1979)
Enriched native	M	1% g		0.13–0.28	0.020–0.065		Goldman et al (1987)
Cultured, unidentified	F	No		0.13–0.21	0.013–0.046		Vadstein (1995)
Enriched native	M	No		0.07–0.26	0.006–0.083		Tezuka (1990)
P. putida	M	No		0.15–0.26	0.016–0.074		Martinussen (1991)
Cultured, unidentified	F	No		0.25			Finlay & Uhlig (1981)
E. coli	F	No	90		0.105		Heldal et al. (1985)
Enriched native	F	No			0.027–0.076		Jürgens & Güde (1990)
Enriched native	M	No	64–136[a]	0.15–0.30	0.037–0.067		Tuomi et al. (1995)
P. halodurans, A. luteo-violaceus	M	No		0.22–0.33		0.010–0.011	Cuhel et al (1981)
C. lividum, P. fluorescens and cultured native	F	No				0.004–0.012	Jordan & Peterson (1978)

[a] Calculated from data in the paper

substrates with a range of N:C values (0.12 to 0.78), yielded in the stationary phase cellular N:C values in the range 0.19 to 0.28 (Goldman et al. 1987). Gräzer-Lampart et al. (1986), who supplied chemostat cultures of *Hyphomicrobium* with a range of reservoir N:C values (0.05 to 0.78), found that when free N could be detected (reservoir N:C higher than ca 0.17) the biomass N:C was approximately 0.29, whereas when free C was available in the medium (reservoir N:C less than ca 0.09) the biomass N:C was approximately 0.19. Intermediate biomass N:C values were found when both N and C sources were depleted from the medium.

Lower N:C ratios were determined for the bacteria from brackish and fresh waters (Table 2); 0.17 (Tvärminne) and 0.20 to 0.22 (Lake Kalandsvatnet and *Escherichia coli*, stationary phase). Zweifel et al. (1993) found a N:C ratio of 0.22 for bacteria from the Baltic Sea, an area in which it has been suggested that bacterial growth may be limited by nitrogen (Lignell et al. 1992). A higher N:C value (0.29) was found for growing cells of *E. coli* (Table 2).

Nitrogen is predominantly associated with protein and nucleic acids, with N:C values of 0.32 and 0.46, respectively (Norland et al. 1995). Cellular N:C values much higher than 0.35 should, therefore, be difficult to envisage. Cells grown under conditions which allow storage of carbon may have low N:C and P:C ratios. Conversely, high N:C and P:C ratios may indicate carbon limitation. Maaloe & Kjeldgaard (1966) have shown that RNA and protein content of bacteria increase with increasing growth rate. The differences found in N:C and P:C values between stationary and growing *Escherichia coli* (Table 2) support such a dependency, whereas the data for *Vibrio natriegens* do not.

The pooled set of data for nitrogen versus carbon content measured at the single-cell level is shown in Fig. 2a, where the dashed line is the regression line and the solid line indicates the Redfield ratio. Table 3 shows the Type II regression equation (Eq. 5) for nitrogen versus carbon based on log-transformed variables. The scale coefficient of Eq. 5 is not significantly different from unity, implying that no size dependence for N:C exists in our data. The pooled coefficient of variation of the N:C value is 0.44.

Phosphorus-to-carbon ratio

The mean phosphorus content of the bacteria from the samples varied from 0.47 to 31 fg cell^{-1} (Table 1). The P:C ratios for the various samples are given in Table 2 and vary from 0.040 to 0.090, with a pooled mean value for all samples of 0.052 (molar C:P = 50:1), which is approximately twice the ratio for P:C given by Redfield et al. (1963) for algae. From the data presented we find that a 'typical' molar C:N:P for bacteria is 50:10:1, a value similar to that found by Goldman et. al. (1987), i.e. 45:9:1. If all phosphorus in the cells were associated with nucleic acids, the mass fraction of nucleic acid in a typical bacterium would be ~20%. Excess phosphorus in bacteria is commonly stored as polyphosphate, but despite the fact that polyphosphate granules are easily seen in unstained bacteria in TEM (Kjeldstad et al. 1991), such granules were rarely observed in the samples included.

In Fig. 2b, phosphorus values are plotted versus carbon for the pooled sample. The dashed line in Fig. 2b shows the regression line for the logarithmically transformed data, while the solid line is the Redfield ratio. A Type II regression equation for P versus C is given in Table 3 as Eq. (6). The scale factor indicates that the largest bacteria have a higher P:C ratio than the smaller ones. The coefficient of variation of the P:C ratio for the pooled samples is 0.58.

Oxygen-to-carbon ratio

The mean oxygen content of the native bacteria varied in the range of 4 to 9 fg cell^{-1} for the samples included. The mean per cell values for both *Vibrio natriegens* and *Escherichia coli* dropped from 120 fg cell^{-1} in the growth phase to 24 fg cell^{-1} in the stationary phase. In Table 2 the mean O:C ratios are listed. For the native bacteria these ratios were in the range from 0.23 (Lake Kalandsvatnet) to 0.77 (Knebel Vig, 1992), with a pooled mean for the all samples of 0.37. Generally, this O:C ratio is too low to be explained within the limits of the accepted composition of the bacterial cell; the O:C of protein is 0.44 and is even higher for nucleic acids and carbohydrates. The discrepancy is probably due to loss of oxygen during analysis (Norland et al. 1995). For *E. coli* we found a marked reduction of the O:C ratio from 0.35 for cells in the growth phase to 0.22 for cells in the stationary phase. In contrast, there was a slight increase in the O:C ratios from 0.34 for cells in the growth phase to 0.39 for cells in the stationary phase for *V. natriegens*. However, lower O:C values in the stationary phase are also found for other cultured bacteria and seem to be a general phenomenon (authors' unpubl. results). The lower O:C ratio of cells in the stationary phase may only partly be explained by their lower levels of phosphorus and sulfur. Similarly, the high O:C ratio found for the sample from Knebel Vig in 1992 (0.77) can partly be explained by high P:C and S:C ratios in this sample.

Data for the single cell measurements of oxygen versus carbon (Fig. 2c) are summarized in the equation for

the Type 2 regression of the O:C relationship (Table 3, Eq. 7). The scale factor of 0.94 suggests that the O:C ratios are higher in small bacteria than in larger ones. One possible explanation for the low oxygen content found in some of these samples may be mass loss of oxygen under the electron beam (Norland et al. 1995). However, since this loss is expected to be dose-dependent, a greater loss should be expected in small bacteria.

The oxygen content of bacteria will give a measure of the oxidation level of the cells. The oxygen fraction of protein, carbohydrates, fats, nucleic acids and polyphosphates are roughly 0.22, 0.53, 0.08, 0.31, and 0.61, respectively (Norland et al. 1995). One of the major intracellular reserve polymers is glycogen, a carbohydrate which may be accumulated in the cells during the stationary phase or under nutrient-limited growth conditions with an excess of carbon sources in the media (Preiss & Romeo 1989). Carbon limitation will most probably lead to a reduced cellular level of carbohydrates (glycogen), fats, nucleic acids (RNA), and protein in this order (Preiss & Romeo 1989 and references therein). The oxygen content of microorganisms is rarely reported in the literature. Luria (1960) reported a mass fraction of 0.2, estimated by difference.

Based on the results presented, we suggest that the O:C ratios of cells may be a signature for the physiological conditions of these cells.

Sulfur-to-carbon ratio

The mean sulfur content for the samples of native bacteria was in the range from 0.30 to 0.56 fg cell^{-1}. For *Vibrio natriegens* and *Escherichia coli* 13 and 9 fg cell^{-1} and 2.5 and 1.7 fg cell^{-1} were found for growing cells and cells in the stationary phase, respectively (Table 1). The mean S:C ratio for pooled set of data is 0.031, with sample means ranging from 0.016 to 0.084 fg cell^{-1} (Table 2). The high CV for the pooled sample is to some extent due to measurement errors caused by the low level of sulfur in the bacteria. The amounts of sulfur measured were close to the detection limit for the method used, and for about 6% of the cells sulfur was below the detection limit. S:C ratios in the range of 0.004 to 0.012 have been reported by others (Jordan & Peterson 1978, Cuhel et al 1981). Cellular protein may contribute to the cellular S:C ratio in the range 0.005 to 0.015 (Jordan & Peterson 1978, Simon & Azam 1989). The high S:C ratio of 0.084 found in the bacteria from Knebel Vig in 1992 may partly be explained by a high cellular content of sulfate, since these cells also showed a high O:C ratio (0.77). Furthermore, a high correlation is found between O:C and S:C values; r = 0.81 for the pooled sample (data not shown), suggesting that sulfur is associated with oxygen, e.g. as sulfate.

The high sulfur content in marine bacteria could be related to uptake of the compatible solute dimethylsulfoniopropionate (DMSP). DMSP is known to be produced by some algae and are metabolized by bacteria (Kiene 1990, Kiene & Bates 1990, Ledyard et al. 1993). Two of the samples, Knebel Vig (1992) and Raunefjorden (June 1993), were taken during blooms of *Prorocentrum minimum* (Prorocentraceae) and *Emiliania huxleyi* (Prymnesiophyceae), respectively, and both these algae are known to produce DMSP (Matrai & Keller 1994). If similar DMSP concentrations as determined in *E. huxleyi* (Malin et al. 1993) occur in marine bacteria, DMSP may contribute up to 0.03 to the bacterial S:C ratio.

For *Escherichia coli*, values of <15 mM cytoplasmic dissolved sulfur were found in cells grown at a salt concentration of 0.5 M NaCl (Cayley et al. 1991). This sulfur content can at the most account for ~0.005 of the S:C ratio. However, the ratio we have determined (0.026) for growing *E. coli* is higher than the sum of the 2 sulfur fractions based on the data from the literature. In addition, low molecular thiols may participate; however, only low values (<5 mM) have been reported (Smirnova & Oktyabrsky 1995) and may explain approximately 0.001 of the S:C ratio.

Sources of variation

Among-group variance

For each of the ratios presented in Table 2, the among-samples coefficient of variation, CV_A, was calculated using Eq. (1), and the values are given in Table 3. The lowest CV_A is found for N:C (0.12), a value substantially lower than the variation seen in the literature data (Table 4). Nitrogen is mainly associated with protein and nucleic acids, macromolecules which make up a large fraction of the cellular material, and this will put constraints on the variability of the N:C value. The CV_A is also low for P:C (0.24). The large difference in relative variation between S:C, which has the highest CV_A (0.61), and N:C shows that a major part of bacterial sulfur is not associated with protein. Furthermore, a high positive correlation between sulfur and oxygen, which has a CV_A of 0.38, suggests that much of this sulfur may be associated with oxygen, e.g. as SO_4^{-2}. A high relative variation (CV_A = 0.38) was also found for the C:volume ratio, a value that, together with the values listed for the same ratio in Table 4, emphasizes the weak foundation upon which the idea of a universal conversion factor from volume to carbon rests.

Within-sample variance

The pooled within-samples coefficient of variation, CV_W, has been calculated using Eq. (2) and the values are given in Table 3. The values are in the range from 0.38 for O:C to 0.60 for S:C. The fact that the CV_W increases as the ratio decreases (Tables 2 & 3) suggests that elements that make up a relatively large part of the cells' dry weight, like oxygen, are more constrained in their relative variation than elements that make up a smaller fraction, like sulfur. The C:volume ratio has a CV_W of 0.46, the next highest of the ratios, implying that within a community a considerable variability in water content exists.

Contribution from measurement error

In contrast to CV_A, CV_W includes random measurement errors. The within-sample variance for ratios is the sum of 3 relative variances: the relative variance associated with measurement of carbon, the relative variance associated with the other variable (volume, nitrogen, etc.), and finally the inherent relative biological variance.

All ratios include carbon as either divisor or dividend. The major uncertainty in the measurement of carbon is the local variation in the thickness of the supporting film (Norland et al. 1995), implying that small bacteria with low mass thickness (i.e. low mass:area) will have a larger error associated with carbon than larger bacteria. This introduces a relative error with a CV of approximately 0.24 for the mean cell and increases to 0.50 for the smaller ones (e.g. Knebel Vig, 1992). About 10% of the bacteria in the pooled sample have a carbon mass thickness below the detection limit for carbon (10 fg C m^{-2}). This error can be reduced by using a thinner (in terms of carbon) and more even supporting film.

The relative errors associated with nitrogen, oxygen, phosphorus and sulfur are mainly determined by counting statistics (Norland et al. 1995) and are less important. Based on counts under the peaks and in their background, relative errors between 0.03 and 0.15 may be estimated. An exception to this was sulfur in the sample from Lake Kalandsvatnet, a sample comprising small bacteria with a low sulfur content, that had a relative error of 0.3. The relative errors of the volume estimates have been discussed above. If the estimated contribution due to these 2 sources of relative error is subtracted, the within-sample CVs listed in Table 3 will be reduced by a value between 0.05 (for S:C) and 0.08 (for O:C).

It can therefore be concluded that most of the relative variation, e.g. seen as scatter around the regression lines in Figs. 1 & 2, is biological heterogeneity and is not an artifact of the measurements. To our knowledge, this is the first time that such an extensive list of within-sample variation, based on measurements of single cells, has been presented.

'Elemental' diversity

The relative variation of the elemental composition within a sample may reflect aspects of the physiological regime in the bacterial community. To obtain an index that expresses this diversity of the elemental composition, we have subjected each sample to a principal components analysis (PCA). PCA will replace the original axes (i.e. measured variables) with new orthogonal axes positioned to maximize the relative variation. This allows us to determine the 'volume' of the hyper-ellipsoid describing the sample. We used the CV corresponding to the radius in a hyper-sphere with the same 'volume' as a diversity index. This index is a CV that expresses how far from the mean vector the bacteria in the sample are. *Vibrio natriegens* and *Escherichia coli* samples had the lowest index, 0.14 and 0.22, and 0.17 and 0.27, for stationary and growing states, respectively. The native samples, however, ranged from 0.42 (Raunefjorden, June) to 0.60 (Tvärminne). Native samples had a significantly higher diversity index than *V. natriegens* and *E. coli*, and samples from the stationary phase had a lower index than growing ones for laboratory-grown samples. A significant part of the heterogeneity seen in our data stems from differences in means between species. These indices should, hence, correlate with traditional species-based indices, e.g. the Shannon index. Communities in stable environments tend to have a higher species diversity than communities exposed to seasonal or periodic perturbations (Odum 1971).

Canonical variate analysis

Canonical variate analysis is a multivariate statistical technique that maximizes the among-group variation relative to total variation. Graphical presentations based on the first 2 canonical axes show the best possible separation of the groups (dispersion). In Fig. 3 sample means of the 10 samples are plotted along with vectors presenting the direction and contribution from the original variables to the dispersion. The first canonical axis, which explains 45% of the dispersion, may be interpreted as a growth or activity axis separating the growing laboratory cultures from the rest. This separation is mainly brought about by differences in levels of nitrogen, phosphorus or sulfur, probably related to rel-

atively more protein and nucleic acids in rapidly growing samples. Stationary-phase laboratory cultures are intermediate between growing laboratory cultures and samples of native bacteria. The second canonical axis, which explains 30% of the dispersion, mainly separates the Knebel Vig (1992) sample with its high levels of oxygen and sulfur from the other samples.

Elemental content and physiological status

The chemical and, thus, the elemental composition of bacteria depends on growth conditions (Maaloe & Kjeldgaard 1966, Gräzer-Lampart et al. 1986). Droop (1983) proposed a kinetic model for growth in which he assumes that the growth rate, μ, depends on a internal surplus pool of nutrients and that it is controlled by the nutrients with a cell quota, Q, which is relatively closest to its minimum, Q_0. The Droop model is given by the equation:

$$\mu = \mu_m\left(1 - \frac{Q_0}{Q}\right) \quad (9)$$

where μ is growth rate and μ_m is maximal growth rate. Cell quota, Q, is expressed either on a per cell basis of element or relative to carbon. Q_0 is the minimum subsistence quota of the limiting factor, below which no growth occurs. On this basis more comprehensive models have been proposed, e.g. describing the relationship between external substrate concentration, internal cell quota and growth rate under steady state conditions (Martinussen & Thingstad 1987, Thingstad 1987, Egli 1991). More than one nutrient may be depleted from the medium, but according to this model only a single nutrient may be limiting growth; other nutrients may, however, control the composition of the biomass (Thingstad 1987).

Using chemostats with a range of N:C values in the reservoir, Gräzer-Lampart et al. (1986) found that when only carbon was depleted from the medium the N:C value in the bacteria was 0.29, while when only nitrogen was depleted the N:C value of the bacteria was 0.18 to 0.20. Intermediate values were found when both nutrients were depleted. Similar values (0.31 and 0.18, respectively) may be calculated from Thingstad (1987). The sample means for N:C values reported in the present paper (Table 2) cover the same range. The above results suggests that the following rule of thumb may be used: if the cellular N:C value is 0.3 or higher, there should be free N in the environment; if the cellular N:C value is below 0.2, the bacterial growth is limited by the N supply. If we apply this to the present data, the bacteria in the sample from Tvärminne were growth-limited by nitrogen.

A wide range of Q_0 for phosphorus has been reported (Vadstein 1995); furthermore, it is well known that bacteria store large amounts of phosphorus when it is available (luxury uptake). This implies that it is more difficult to make statements about physiological status based on cellular content for this element. However, if we use a 'typical' Q_0 P:C value (Vadstein 1995) of 0.031, and if we assume that the native bacteria had a growth rate well below μ_m, we may conclude that the bacteria in our native samples were not P limited. A possible exception might be the sample from Raunefjord, October, which had a P:C value of 0.041. This sample showed a clear growth response following phosphorus addition (Tuomi et al. 1995).

Normally, N:C values and especially P:C values are high for native bacteria (Table 2). Egli (1991) has shown that slow-growing bacteria have a high carbon demand. This suggests that the supply of carbon will control the bacterial composition, and even often limit their growth.

It has been shown by laboratory studies of bacterial growth that chemical composition and cell size are dependent on growth rate (Maaloe & Kjeldgaard 1966, Ingraham et al. 1983). In the discussion above we suggest that the relative cellular content of carbon, nitrogen and phosphorus may point to which element is limiting bacterial growth or is controlling bacterial composition. Neither the data for elemental composition presented here nor data from the literature, however, are particularly useful when it comes to estimating activity or growth rates. Our preliminary results, which suggest that the relative oxygen content is lower in stationary state cells than in exponential growing cells, will be pursued. A wider spectrum of elements than those commonly dealt with (C, N and P) is required to approach the issue of the relationship between elemental composition and bacterial activity.

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