Limnol. Oceanogr., 48(5), 2003, 1732–1743

Elemental composition of single cells of various strains of marine *Prochlorococcus* and *Synechococcus* using X-ray microanalysis

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Abstract

We measured the elemental composition of single cells from six strains of marine *Prochlorococcus* and two strains of marine *Synechococcus* by X-ray microanalysis in the transmission electron microscope (TEM), which allows measurements of all major elements in cells without any fixation or staining. The mean carbon : volume ratios ranged from 136 to 280 fg μm^{-3} in *Prochlorococcus* and 138 to 290 fg μm^{-3} in *Synechococcus.* Using mean values of elemental content of C : N : O : P : S, we obtained molar ratios of 143–214 : 15–24 : 15–41 : 1 : 0.58–1.64, and 65–293 : 7–36 : 11–36 : 1 : 0.31–1.54 for strains of *Prochlorococcus* and *Synechococcus,* respectively. The diffusible ions Mg^{++} (89–490 mmol L^{-1}) and Na^{+} (230–660 mmol L^{-1}) dominated in *Prochlorococcus.* In *Synechococcus* Na^{+} (90–480 mmol L^{-1}) dominated. For all samples the range of K^{+} content was 23–130 mmol L^{-1}. Elemental composition varied widely in relation to strains and growth media. However, C : P and N : P ratios were above the Redfield ratio in all *Prochlorococcus* strains and one of the *Synechococcus* strains (WH 8103). This likely reflected the low P content of these cells. This low P requirement would be clearly advantageous in the oligotrophic conditions occupied by these organisms. The high relative carbon content, compared to what has been found for heterotrophic bacteria using similar methods, is suggested to confer a competitive advantage to photosynthetic over heterotrophic bacteria in ecosystems where both functional groups are mineral nutrient limited.

Marine cyanobacteria of the genera *Synechococcus* and *Prochlorococcus* comprise the prokaryotic component of the oxygenic photosynthetic picoplankton. These <2-μm sized cells contribute around half of the carbon fixed in marine systems and hence are of particular ecological significance with regard to global C cycling (*see* Partensky et al. 1999*a*). Since the first reports describing the presence of *Synechococcus* and *Prochlorococcus* in the euphotic zone (Johnson and Sieburth 1979; Waterbury et al. 1979; Chisholm et al. 1988), much has been learned of the physiology and molecular ecology of these organisms (*see* Carr and Mann 1994; Partensky et al. 1999*b*; Moore et al. 2002; West and Scanlan 2002; Scanlan 2003). However, one important area that has largely escaped investigation, despite its potential ecophysiological significance, has been the C : N : P stoichiometry of these organisms. Indeed, relatively few reports on elemental composition of marine bacteria are available, and for *Synechococcus* and *Prochlorococcus* the only reported data are from carbon and/or nitrogen measurements of bulk preparations (Cuhel and Waterbury 1984). Here we report on the cellular content of macroelements in two strains of *Synechococcus* and six strains of *Prochlorococcus.* Our measurements are based on single-cell analysis in the transmission electron microscope (TEM), and we can thus avoid including contaminating bacteria or extracellular material not related to specific cells.

The use of TEM–X-ray microanalysis for quantitative analyses of elemental content in single cells has been previously reported both for cultured heterotrophic bacterial cells (Heldal et al. 1985; Norland et al. 1995; Oren et al. 1997) and cells from natural marine environments (Fagerbakke et al. 1996). Most other reports have used methods based either on bulk analysis of C and N for the bacterial fraction (Fukuda et al. 1998) or on flow cytometer measurement of the protein content of single cells (Zubkov et al. 1999). From X-ray microanalyses of individual marine heterotrophic cells a molar C : N : P ratio of 50 : 10 : 1 has been found (Fagerbakke et al. 1996), which indicates a higher content of N and P in bacteria, relative to C content, compared to algae, which has an average ratio of 106 : 16 : 1 (Redfield 1958). In the work presented here strains of the marine cyanobacterial genera *Synechococcus* and *Prochlorococcus* show C : N : P stoichiometries of 65–293 : 7–36 : 1

Acknowledgments

We thank Egil S. Erichsen, Laboratory for Electron Microscopy, University of Bergen, for his contribution. This study was in part financed by the EC through grants CYCLOPS (EVK3-CT-1999-00009) to F.T. and MARGENES (QLRT-2001-01226) to D.J.S. D.J.S is a Royal Society University Research Fellow.

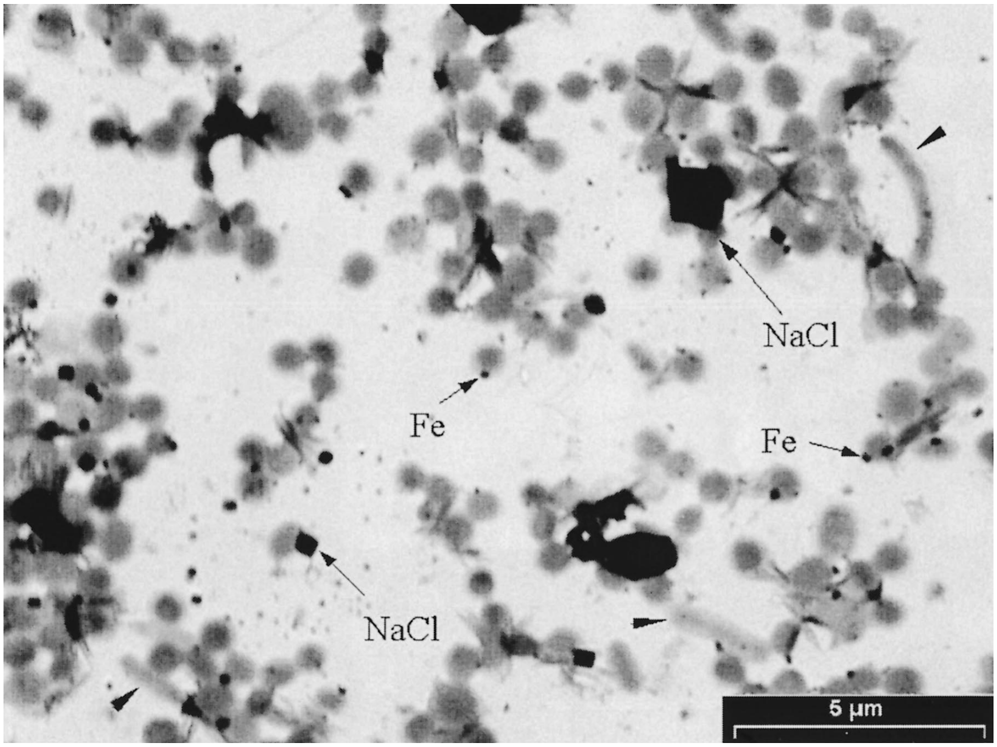

Fig. 1. Electron micrograph of *Prochlorococcus* cells (NATL1-MIT) harvested by centrifugation onto Al grids, supported with carbon-coated formvar film, and air dried. Both *Prochlorococcus* and contaminating bacteria (arrow heads) are shown. Some precipitations of NaCl and iron rich precipitations on the cells are marked.

and 143–214 : 15–24 : 1, respectively. The high relative C content of the cells may be interpreted as a storage of C for energy-deficient situations. We argue, however, that the volume increase possible with a high C content may be of competitive advantage at low external concentrations of, e.g., phosphate.

Materials and methods

Cells and growth conditions—The axenic *Synechococcus* strains WH 8103 and WH 7803 were grown in batch culture under constant illumination (10 μmol photons m^{-2} s^{-1}) at 25°C in three different growth media: (1) a chemically defined artificial seawater medium (ASW) (*see* Wilson et al. 1996) (2) ASW with the [Mg^{++}] reduced to 10 mmol L^{-1}, and (3) a seawater based medium PCR-S11 normally used for growth of *Prochlorococcus* (*see* Rippka et al. 2000). Cultures were maintained for several generations in the respective media before collecting cells for analysis. The N and P content of ASW and PCR-S11 medium are 174 μmol L^{-1} P, 8.8 mmol L^{-1} N and 50 μmol L^{-1} P, 400 μmol L^{-1} N, respectively.

The axenic *Prochlorococcus* strain PCC 9511 [HLI], obtained from the Pasteur culture collection of cyanobacteria (PCC) (Institut Pasteur), and the unialgal *Prochlorococcus* strains EQPAC1 [HLI], SB [HLII], GP2 [HLII], SARG [LL], and NATL1-MIT [LL] obtained from the culture collection of the Station Biologique, Roscoff, France, were grown in batch culture under continuous illumination (35 μmol photons m^{-2} s^{-1}) at 22°C in PCR-S11 medium (Rippka et al. 2000). HLI, HLII, and LL refer to the previously designated high light (HL) adapted and low light (LL) adapted phylogenetic clades that these strains represent examples of (*see* West and Scanlan 1999).

All the cultures were harvested in the late exponential phase of growth. Normally we harvest native marine bacteria as discrete particles on the grids, which have a hydrophobic surface due to the carbon coating. Cultured cells are generally found to aggregate in clusters on the grid surface either due to the way they grow or as a result of centrifugation/air drying. All the samples analyzed in this series are from clusters of cells, and our preparations were virtually without cells lying as discrete particles on the grids.

Elemental analyses of single marine cyanobacterial cells in the electron microscope—Cells were collected by centrifugation in a Beckman model L8-70M preparative ultracentrifuge, using a SW41 swing-out rotor for 10 min, at 10,000 rpm (7000 × g) at 20°C on aluminum grids (100 mesh, Agar Scientific) supported with a carbon-coated formvar film and then air dried. Cells were viewed and analyzed for elements in a Philips CM 200 electron microscope. The sample grids were mounted between beryllium rings. The microscope was operated in scanning mode at a tilt angle of 38°, 80 kV acceleration voltage, magnification between 5,000 and 10,000, spot size of 14 nm (spot size 3), and an accumulation time (live time) of 30 s. The X-ray spectrum was recorded from an area that circumscribes the specimen (Norland et al.

Table 1. Size and cellular content of carbon, nitrogen, oxygen, phosphorus, and sulphur measured in single cells of laboratory grown strains of *Prochlorococcus.*

Strain	Volume (μm^3)	C/vol (fg μm^{-3})	C (fg)	N (fg)	O (fg)	P (fg)	S (fg)	*n**
SARG	0.144±0.008†	237±7	34±2	4.5±0.2	6.4±0.4	0.41±0.02	0.33±0.02	29
NATLI-MIT	0.139±0.010	246±8	33±2	4.3±0.2	5.9±0.3	0.42±0.02	0.36±0.02	25
GP2	0.13±0.01	136±9	17±2	2.2±0.2	5.3±0.6	0.25±0.03	0.29±0.05	22
SB	0.22±0.01	147±5	32±2	3.7±0.2	9.6±0.7	0.56±0.06	0.39±0.02	39
EQPAC1	0.077±0.005	280±10	21±1	2.9±0.2	3.0±0.2	0.38±0.04	0.23±0.01	25
PCC 9511	0.22±0.02	158±7	34±3	4.3±0.4	7.4±0.6	0.49±0.04	0.83±0.07	26

* Number of cells analyzed.
† SE.

1995). The light element detection system consisted of EDAX detector DX-4 supported with SIS soft imaging software. In this system we identify cells by the imaging system, the cell area is marked out, and an identical area is located in a particle-free area. The scanning mode of the electron beam is run through the imaging system. For each preparation, between 15 and 35 cells were analyzed. To quantify the contribution of the supporting film, we recorded the spectra of particle-free area of identical size and shape adjacent to each cell. Carbon measurements were calibrated using latex beads of known size (Agar Scientific), and calibration constants for the different elements were determined according to Norland et al. (1995).

The X-ray data and measurements of cell size (logarithmic values) were subjected to canonical variate analysis using standard software (Statistica 6.0, Statsoft). Each strain of *Prochlorococcus* and the strains/growth conditions for the *Synechococcus* samples were treated as separate groups. The canonical variates are linear combinations of the original (logarithmic) values, where the ratio between among-group variation and total variation is maximized.

Results

An electron micrograph of *Prochlorococcus* NATL1-MIT cells plus contaminating bacteria is shown in Fig. 1. Sodium chloride and iron rich precipitations on the surface of the *Prochlorococcus* cells can be clearly seen and were also observed in other *Prochlorococcus* strains investigated in this study.

Size and cellular content data—of carbon, nitrogen, oxygen, phosphorus, and sulfur—for six different *Prochlorococcus* strains are presented in Table 1. The mean volumes of cells from various strains of *Prochlorococcus* in our air-dried preparations are in the range of 0.08 μm^3 to 0.22 μm^3. The C : volume ratios for *Prochlorococcus* are in the range of 136 fg C μm^3 to 280 fg C μm^3, which is on average considerably higher than the 30–148 fg C μm^3 that have been reported for native marine bacteria (Fagerbakke et al. 1996; Gundersen et al. 2002). Elemental ratios of C : N, C : P, N : P, C : O, and C : S for the same *Prochlorococcus* strains are summarized in Table 2. The C : N and C : P ratios are fairly stable for this group of strains, C : N 8.5–9.9 mol/mol and C : P 160–215 mol/mol, respectively. As shown in Fig. 2, C : N of the cells included in this series of analyses are higher than the Redfield ratio. Moreover, the N : P ratios for these cells are in the range of 16–24 mol/mol (Table 2). Thus, strains representative of all the different phylogenetic groups of *Prochlorococcus* analyzed here have N : P ratios above the Redfield ratio (N : P = 16). In fact the N : P values presented in Table 2 show that within each group of cells analyzed there is as broad a distribution (Fig. 3) as that reported for all mean values of N : P ratios found in other phytoplankton (Geider and La Roche 2002). This is also indicated by the distribution of single-cell measurements shown in Fig. 2. All these ratios point in the same direction: higher C : N, C : P, and N : P ratios than those given in the Redfield ratio (Redfield 1958; Goldman et al. 1979). Further, we did not observe any structural signs of polyphosphate bodies in the *Prochlorococcus* strains included in this study, which suggests these organisms have a low P-storage capacity.

The sulfur content of the cells is variable, as seen from the C : S values (Table 2), which range between 111 and 276 mol/mol. This is somewhat higher than the average C : S for marine heterotrophic bacteria (~85, Fagerbakke et al. 1996). It is possible that the content of nonprotein sulfur is low in

Table 2. Elemental ratios (mol/mol) of *Prochlorococcus.* The number of cells analyzed was the same as for Table 1. All numbers are given as mean ratios of estimates per individual cell.

Srain	C : N	C : P	N : P	C : O	C : S
SARG	8.8±0.1*	215±9	24.4±0.9	7.3±0.3	276±8
NATLI-MIT	9.2±0.2	206±7	22.6±0.7	7.6±0.1	260±10
GP2	9.2±0.2	190±10	21±1	4.4±0.1	180±10
SB	9.9±0.2	156±6	15.9±0.6	4.49±0.09	220±7
EQPAC1	8.5±0.2	160±10	19±2	9.8±0.4	250±10
PCC 9511	9.5±0.2	190±10	20±1	6.2±0.2	111±4

* SE.

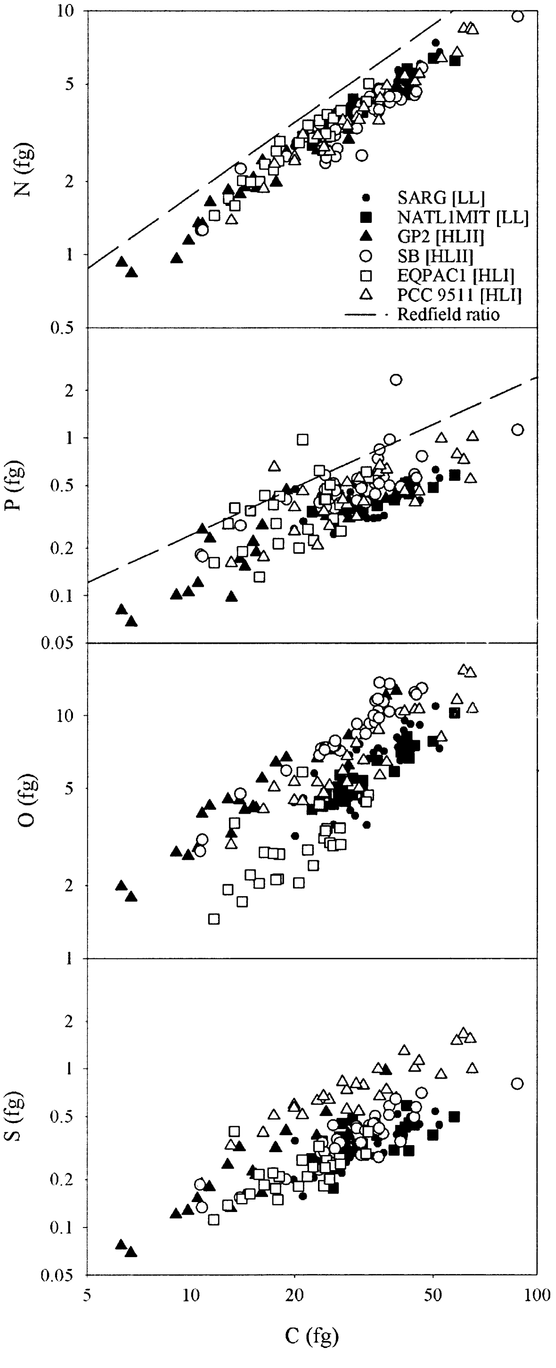

Fig. 2. Single-cell measurements of N, P, O, and S versus C for six *Prochlorococcus* strains.

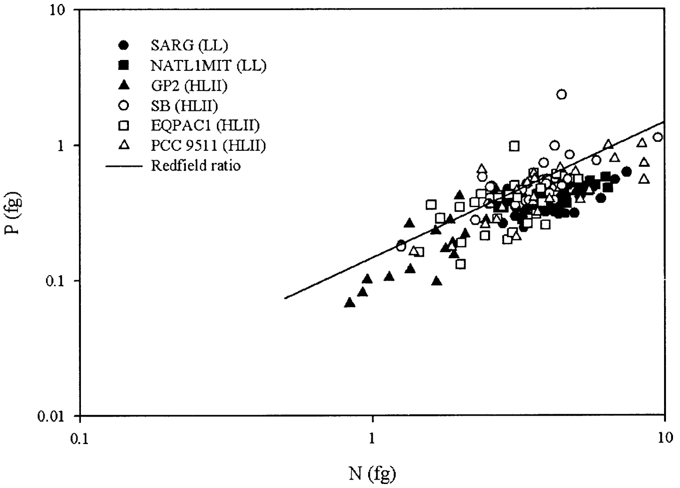

Fig. 3. The cellular content of P versus N for *Prochlorococcus.*

these *Prochlorococcus* cells. For some marine bacteria the nonprotein sulfur content has been estimated to be about 20% (Cuhel et al. 1981) and in some algae significantly higher, mainly in compounds such as dimethylsulfide (DMS) and dimethylsulfoniopropionate (DMSP) (Matrai and Keller 1994).

The C : O ratios of these groups of cells were in the range of 4.4–9.8 mol/mol. As seen from Table 2, the values were 4.4 and 4.5 for the *Prochlorococcus* strains GP2 and SB, while the other strains had C : O ratios between 6.2 and 9.8. These differences may reflect intrinsic differences in the elemental composition of strains SB and GP2, which are representatives of the HLII clade, or reflect subtle differences in the growth state of the cells. From Fig. 2 the contrast in O level is clearly demonstrated for strains EQPAC1 and GP2, but this difference is not statistically significant as indicated by canonic variate analysis for the whole data set (data not shown).

The cellular content of sodium (Na^+), magnesium (Mg^{++}), chlorine (Cl^-), potassium (K^+), and calcium (Ca^{++}) in these *Prochlorococcus* strains is presented in Table 3. The Na^+ content of the cells was relatively high (300–660 mmol L^{-1}) compared to the K^+ content (23–64 mmol L^{-1}), and *Prochlorococcus* strains PCC 9511 and SB possessed the highest Na^+ concentrations, 550 mmol L^{-1} and 660 mmol L^{-1}, respectively. For the other strains the Na^+ content was about 300 mmol L^{-1} or lower. The K^+/Na^+ ratio was in the range of 0.07–0.21 as calculated from the mean values, but considerably higher for some of the individual cells measured. In Fig. 4 the single-cell estimates for the cations were plotted against Cl^- concentrations, and for most of the strains analyzed there is a positive correlation between Cl^- and the cations. Figure 4 also demonstrates the low Mg^{++} content and high Na^+ content of strain PCC 9511, as shown in Table 3. Magnesium was the dominating cation in most of the *Prochlorococcus* strains analyzed. Strain PCC 9511 had 89 mmol L^{-1} Mg^{++}, while the other strains contained between 370 and 520 mmol L^{-1}. As seen from both Table 3 and Fig. 4, strain PCC 9511 appears as a well-defined group of cells as a function of its Mg^{++} content. The only anion analyzed in this series was Cl^-, whose content ranged between 100 and 270 mmol L^{-1} in those *Prochlorococcus* strains analyzed. Thus there is a much higher cellular content of cations than that of Cl^- in these organisms.

The Ca^{++} content of the cells is in general low, 15–50 mmol L^{-1}, compared to the Mg^{++} content, with a maximum value of 170 mmol L^{-1} Ca^{++} for the data set as a whole.

Analyses of carbon, nitrogen, oxygen, phosphorus, and sulfur in cells from two strains of *Synechococcus* grown in

Table 3. Estimated concentration of sodium, magnesium, chlorine, potassium, and calcium in cells of *Prochlorococcus.* The number of cells analyzed was the same as for Table 1.

Strain	$[Na^+]$*	$[Mg^{++}]$	$[Cl^-]$	$[K^+]$	$[Ca^{++}]$
SARG	300±20†	390±10	160±20	64±2	18±2
NATLI-MIT	300±10	367±10	185±6	56±2	18±2
GP2	230±20	520±30	172±10	43±3	15±2
SB	550±10	370±10	98±3	63±2	49±2
EQPAC1	310±40	490±20	270±10	23±1	24±7
PCC 9511	660±30	89±4	150±7	44±2	23.5±0.9

* mmol L^{-1}.
† SE.

various media are summarized in Table 4. In total 15 to 36 cells are included in these analyses, and we measured cell volumes in the range of 0.62 μm^3 to 1.56 μm^3. Cells from the two strains and three growth conditions contained between 17 and 36 fg nitrogen per cell and 2.2 and 7.9 fg phosphorus per cell. As seen from Table 4 each group of cells is relatively homogeneous with regard to their content of these elements. The C : volume ratio varied from 138 to 290 fg C μm^3 without any clear-cut correlation between strains and growth media. These C : volume ratios are relatively high compared to other marine bacteria (Fagerbakke et al. 1996; Gundersen et al. 2002). The elemental ratios of C : N, C : P, N : P, C : O, and C : S for this set of samples are summarized in Table 5 and Fig. 5. The C : N ratios were fairly stable and were between 8.2 and 10 mol/mol. The molar ratio of C : N measured for individual cells is shown in Fig. 5 for the whole set of data. The mean value for this data set is 8.7, which is significantly higher than the Redfield ratio (6.6). For the various growth conditions used (10 mmol L^{-1} Mg^{++} ASW, ASW, PCR-S11) the C : P ratio of *Synechococcus* strain WH 7803 was lower (73, 94, 113 mol/mol) than the C : P ratio for strain WH 8103 (350, 270, 150 mol/mol). Indeed, *Synechococcus* strain WH 7803 grown in artificial seawater media showed a C : P ratio below that of Redfield, while strain WH 8103 grown in both ASW and ASW at low Mg^{++} had very high C : P ratios, 270 and 350, respectively. A similar trend is shown for the N : P ratio of these two groups of cells (36 and 43, Table 5). Since the mean C : N ratio for these cells is 8.2, these results may reflect a low phosphorus content of strain WH 8103. The N : P ratios of the individual *Synechococcus* cells measured are shown in Fig. 6. Growth of strain WH 8103 in ASW and ASW 10 mmol L^{-1} Mg^{++}, in which the higher mean N : P ratios are seen, may be a reflection of the bimodal distribution of the N : P ratio seen in this strain as compared to strain WH 7803. The average N : P ratios (mol/mol) for the two *Synechococcus* strains used here and in all growth conditions varied from 8.3 to 43 with a mean value of 21.

The content of inorganic ions of *Synechococcus* is shown in Table 6 and Fig. 7. Both strains WH 7803 and WH 8103 had a lower Mg^{++} content (83 mmol L^{-1} and 70 mmol L^{-1}) and a higher content of Ca^{++} (87 mmol L^{-1} and 73 mmol L^{-1}) in cells grown at low Mg^{++} (10 mmol L^{-1}) compared to cells grown in ASW (the latter is equivalent to the seawater concentration of Mg^{++} [50 mmol L^{-1}]). This could indicate a general need for divalent cations in these cells. Na^+ was the dominant ion found, and mean values ranged from 90 to 480 mmol L^{-1} for the various groups of cells analyzed (Table 6, Fig. 7). The variability in Na^+ content of these cells was relatively low, with coefficients of variation in the range of 0.19–0.60. For Mg^{++} we found mean cellular contents between 70 and 138 mmol L^{-1} for the six groups of cells with 218 mmol L^{-1} as the highest value for the whole data set. The K^+ content of the two *Synechococcus* strains ranged from 45 to 130 mmol L^{-1}. Based on these mean values, the K^+/Na^+ ratio thus varied between 0.12 and 0.64. Whether and how this ratio is related to the activity of the cells is still uncertain. The Cl^- content of these cells was relatively low and constant, 92 to 156 mmol L^{-1}, while values found for individual cells fell between 30 and 250 mmol L^{-1}.

The distribution of Na^+ is clearly bimodal in relation to the cellular content of Cl^- for strain WH 8103 grown in PCRS-11. For WH 8103 grown at 10 mmol L^{-1} Mg^{++}, we also find a higher cellular content of Na^+ (mean 476 mmol L^{-1}) than for the other groups of cells analyzed (Fig. 7). However, no concomitant change in ion composition is observed for strain WH 7803 grown at 10 mmol L^{-1} Mg^{++} (*see Table 2*).

Discussion

Prochlorococcus—The measured cell volumes for the *Prochlorococcus* strains analyzed in this study were found to be in the range of 0.08 to 0.22 μm^3. From other studies we have no evidence for any severe shrinkage of cells during air drying of unfixed material (Norland et al. 1995). Since these cells were harvested directly onto the grids by centrifugation, any signs of shrinkage are seen as open areas along the edges of cells, and this is commonly observed for larger cells during air drying. We estimate an average of 30 fg C $cell^{-1}$ for *Prochlorococcus,* which is somewhat lower than the range of 46–61 fg C $cell^{-1}$ reported by Bertilsson et al. (2003) and 53 fg C $cell^{-1}$ as estimated by Campbell et al. (1994). Recently Claustre et al. (2002) reported on diel variations in *Prochlorococcus* optical properties. In this study the C content for the axenic strain PCC 9511 had a mean value of 27 ± 6 fg C $cell^{-1}$ (range 17–38 fg C $cell^{-1}$). These values correspond very well with those we report here. Claustre et al. (2002) also show a near twofold change in C content during the diel light–dark cycle, a point that may explain the different values reported by other workers.

The C : volume ratio for *Prochlorococcus* ranged from 130

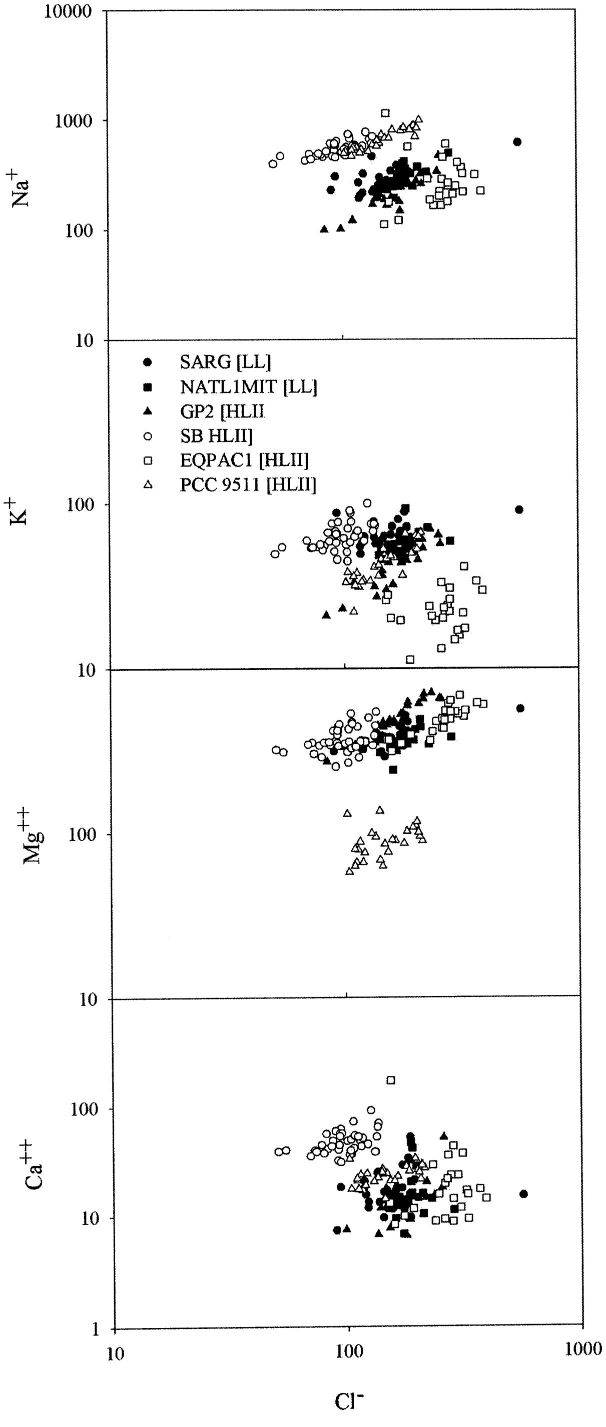

Fig. 4. Estimated cellular content of diffusible ions, Na^+, K^+, Mg^{2+}, and Ca^{2+} plotted versus Cl^- for the analyzed *Prochlorococcus* cells.

fg C μm^{-3} to 250 fg C μm^{-3}, and this is on average considerably higher than the 30–148 fg C μm^{-3} values that have been reported for native marine bacteria by use of X-ray microanalysis (XMRA) (Fagerbakke et al. 1996; Gundersen et al. 2002). For marine bacteria, C : volume ratios of 160–400 fg C μm^{-3} have been reported (Simon and Azam 1989), and other reports on unfixed marine bacteria give values of 155–292 fg C μm^{-3} (Kogure and Koike 1987). For four strains of heterotrophic marine bacteria isolated from the exponential phase of growth, an average C : volume ratio of 148 fg C μm^3 was found (range 52–241 fg C μm^3) (Vrede et al. 2002).

As shown in Table 2, the C : N and C : P ratios of 8.5–9.9 mol/mol and 160–215 mol/mol, respectively, are well above the Redfield ratio (6.6 and 116). The N : P ratios for these cells are in the range of 16–24 mol/mol. These N : P ratios are considerably higher than the range reported for four strains of heterotrophic marine bacteria isolated from the exponential phase of growth, 5.5–7.9, but similar to N : P ratios of 16–21 reported for P-limited bacteria (Vrede et al. 2002). Thus a relatively low P content of *Prochlorococcus* seems to be a general conclusion for the strains involved in this study. Bertilsson et al. (2003) report C : P and N : P ratios of 121 and 21, respectively, for *Prochlorococcus* MED4 and conclude that *Prochlorococcus* has low requirements for phosphorus. Our results thus agree well with these findings. The low P requirement of *Prochlorococcus* may explain the lack of polyphosphate formation in the cells analyzed in this study. It appears, though, that the capacity for polyphosphate synthesis exists in the genus since the gene encoding polyphosphate kinase can be found in both completely sequenced *Prochlorococcus* genomes (*see* http://www.jgi.doe.gov/JGI_microbial/html/index.html).

C : S ratios of 111 to 276 among the six *Prochlorococcus* strains represent a relatively broad range, but even the low C : S value of 111 found for strain PCC 9511 is still well above the C : S ratio of 32 reported for marine heterotrophic bacteria, even though those cells had a low C : volume content (Fagerbakke et al. 1996). In other studies, C : S ratios of 83 to 250 have been reported (Jordan and Peterson 1978; Cuhel et al. 1981). There are two main fractions of sulfur-containing compounds in cells, either the protein fraction (1.1% S w/w) or in compounds related to osmoregulation of cells. We conclude that *Prochlorococcus,* during growth in rich media, does not use any sulfur-containing compatible solutes for osmoregulation.

C : O ratios are rarely reported in the literature, and Luria (1960) reported a mass fraction of 0.2 for bacteria, estimated by difference. We estimate the mass fraction of O in these samples to be about 0.10–0.20, which for some of these strains is markedly lower than that expected according to Luria (1960). For *Escherichia coli,* a marked difference in C : O ratios between exponentially growing (C : O = 2.8) and stationary phase cells (C : O = 4.5) has been reported (Fagerbakke et al. 1996). Since we sampled from cultures at the late exponential phase/early stationary phase of growth, the differences seen for C : O ratios, 4.4 and 4.5 for the *Prochlorococcus* strains GP2 and SB and 6.2 to 9.8 for the other strains, could relate to variable stages of growth. The relatively low level of oxygen might also originate from loss of O during the analysis. If so, the loss of O should be dose dependent and accordingly higher for small cells than larger ones. As reported by Norland et al. (1995), however, no correlation of cell size with O content has thus far been observed. It should also be noted that X-ray microanalysis of calcite (coccoliths from *Emiliania huxleyi*) did not indicate

Table 4. Size and cellular content of carbon, nitrogen, oxygen, phosphorus, and sulphur measured in single cells of two strains of *Synechococcus* sp. during growth in different media.

Strain and growth medium	Volume (μm^3)	C/vol (pg μm^{-3})	C (fg)	N (fg)	O (fg)	P (fg)	S (fg)	n*
WH 7803, 10 mmol L^{-1} Mg^{++} ASW	1.6±0.2†	138±9	200±20	26±2	45±4	7.9±0.8	2.5±0.2	17
WH 8103, 10 mmol L^{-1} Mg^{++} ASW	1.02±0.07	252±8	250±20	36±3	41±3	2.2±0.2	3.5±0.2	36
WH 7803, ASW	0.62±0.07	190±10	120±10	17±2	21±2	3.2±0.3	1.3±0.2	15
WH 8103, ASW	0.83±0.06	290±10	220±10	32±2	38±3	3.6±0.5	2.6±0.2	34
WH 7803, PCRS-11	0.71±0.08	200±10	130±10	18±2	27±3	3.1±0.3	2.3±0.3	20
WH 8103, PCRS-11	1.2±0.2	150±30	150±20	18±3	22±4	2.6±0.4	1.6±0.3	15

* Number of cells analyzed.
† SE.

any specific loss of O during the analyses (Fagerbakke et al. 1994).

All these data point toward a generally high carbon content for *Prochlorococcus.* Perhaps the only explanation for this, if this high C content is related to a specific macromolecule fraction, might be a high content of lipid. Lipids have a relative C : O composition of 0.80 : 0.08 (w/w), while a high level of any other macromolecule fraction, e.g., protein, nucleic acid, carbohydrate or polyphosphate, would influence the other elemental ratios presented above.

The high Na^+ content of the *Prochlorococcus* cells (300–660 mmol L^{-1}) compared to the K^+ content (23–64 mmol L^{-1}) has been observed for other marine bacteria (Fagerbakke et al. 1999). For some bacteria the K^+/Na^+ ratio has been used as a signature of viability or activity in the sense that active (growing) cells have a higher ratio than dormant/dead cells. For exponentially growing bacteria, reported $K^+ : Na^+$ ratios >1 are typical (Fagerbakke et al. 1999). Since in this study we find average values of 0.07–0.21 for the $K^+ : Na^+$ ratios, but much wider ranges are found among individual cells, it is likely that some of these cells are nongrowing or dormant. Alternatively, this high Na^+ content of *Prochlorococcus* cells could be a result of salt precipitated outside the cells due to the high concentration (500 mmol L^{-1}) of Na^+ in seawater. The low K^+ content, on the other hand, could be underestimated due to leakage of K^+ from the cells during the centrifugation and air-drying stages of preparation, but so far we have no evidence for such a loss factor.

With the exception of strain PCC 9511, the Mg^{++} content of the cells was on average 370 to 520 mmol L^{-1} and thus in the range seen for other marine bacteria (Fagerbakke et al. 1999). For strain PCC 9511 we found a mean of 89 mmol L^{-1} Mg^{++}. It is very unlikely that this difference represents a fundamental difference in physiology between the strains. More probably it reflects a shift in ion composition due to slight variations in growth stage of the harvested cells. Further work will seek to clarify this possibility.

The only anion analyzed in this series was chlorine, which ranged between 100 and 270 mmol L^{-1} in the *Prochlorococcus* strains under study. Thus, there is a considerably higher cellular content of cations than that of chlorine. It should also be noted that the relatively high Na^+ content of the cells would result in a high Cl^- content if much of the Na^+ was from NaCl outside the cells. As shown in Fig. 1, it is relatively simple to avoid areas that are influenced by NaCl crystals. In Fig. 8 a plot of canonical variate analysis summarizes the total dispersion of the strains included in the *Prochlorococcus* samples. The two axes shown in Fig. 8 explain nearly 91% of the total dispersion. Canonical variable 1 (67%) reflects mainly the contrast between Mg^{++} and P, C, area, N; while canonical variable 2 (24%) is mainly based on K^+, Na^+ versus Cl^-, N, P, S. Of the six strains included here, the two strains SARG (LL) and NATL1-MIT (LL) were indistinguishable by this analysis. All remaining differences between strains were significant. It is interesting to note that among the four strains, two belong to the HLI cluster and two to the HLII cluster, but all are significantly different from each other. Since these cells were grown in the same media, the differences seen could be related to variation in growth stages, or some of the differences could be genotype specific.

Table 5. Elemental ratios (mol/mol) for *Synechococcus* strains WH 8103 and WH 7803. The number of cells analyzed is the same as for Table 4. All numbers are given as mean ratios of estimates per individual cell.

Strain/growth condition	C : N	C : P	N : P	C : O	C : S
WH 7803, 10 mmol L^{-1} Mg^{++} ASW	9.1±0.2*	73±8	8±1	6.1±0.3	218±7
WH 8103, 10 mmol L^{-1} Mg^{++} ASW	8.19±0.09	350±30	43±5	8.5±0.3	195±4
WH 7803 ASW	8.4±0.1	94±5	11.3±0.7	7.1±0.2	238±7
WH 8103 ASW	8.2±0.3	270±40	36±5	8.2±0.3	239±9
WH 7803 PCRS-11	8.9±0.6	113±5	13.3±0.8	6.9±0.3	200±20
WH 8103 PCRS-11	10.0±0.3	150±10	15±1	9.3±0.3	290±20

* SE.

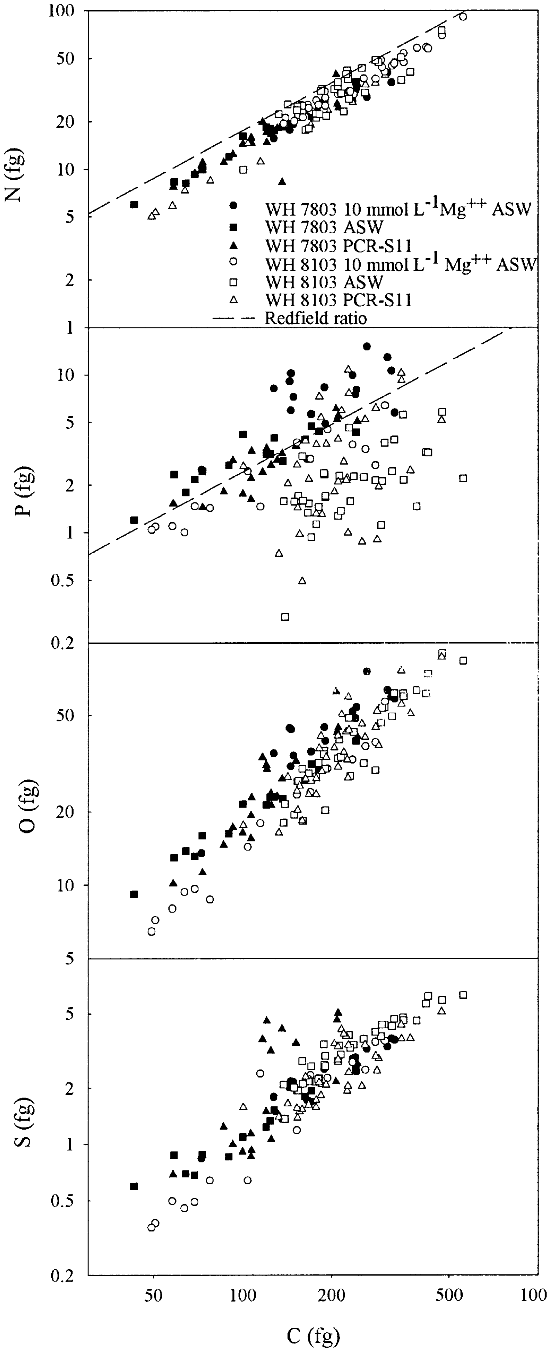

Fig. 5. Single-cell measurements of N, P, O, and S versus C for the *Synechococcus* strains WH 7803 and WH 8103 grown in different media.

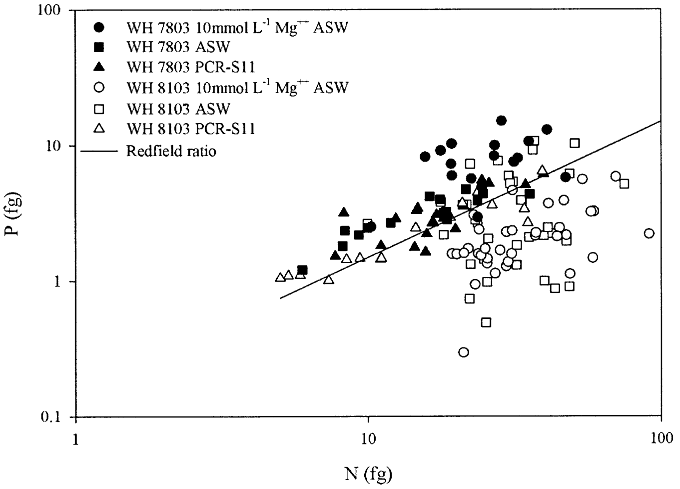

Fig. 6. The cellular content of P versus N for *Synechococcus.*

Synechococcus—As shown in Table 4, there is a marked difference in calculated cell volumes for strain WH 7803 grown in various media. A mean cell volume of 1.62 μm^3 was found for cells grown in 10 mmol L^{-1} Mg^{++} ASW medium, while those grown in PCRS-11 and ASW medium showed mean volumes of 0.71 μm^3 and 0.62 μm^3, respectively. The C : volume ratio was markedly lower for cells grown at 10 mmol L^{-1} Mg^{++} (138 fg μm^{-3}) compared to 200 fg μm^{-3} and 190 fg μm^{-3} for cells grown in PCRS-11 and ASW media. On the other hand, elemental ratios like C : N, C : P, C : O, and C : S were fairly similar for the different growth media used. The mean volumes of strain WH 8103 were in the range of 0.83 μm^3 to 1.2 μm^3, but the C : volume ratio varied from 150 fg μm^{-3} for cells grown in PCRS-11 to 252 fg μm^{-3} and 290 fg μm^{-3} for cells grown in 10 mmol L^{-1} Mg^{++} ASW and in ASW medium. *Synechococcus* strain WH 7803 grown in artificial seawater media showed a C : P ratio below that of Redfield, while strain WH 8103 grown in both ASW and ASW at low Mg had very high C : P ratios, 270 and 350, respectively. A similar trend is shown for the N : P ratio of these two groups of cells (36 and 43, Table 5). Bertilsson et al. (2003) found a N : P ratio of 33 for exponentially growing cells and 110 for P-limited cells of *Synechococcus* WH 8103 and C : P ratios of 165 and 779 at the two different growth conditions, respectively.

In cultures of WH 7803 we observed relatively large amounts of polyphosphate in the cells, and this is likely the main reason for C : P values below the Redfield ratio of 106.

There is a considerable plasticity in the C : N ratio of these two *Synechococcus* strains. A mean C : N ratio of 8.7 for all individual cells measured and ratios between 7.8 and 9.5 (mol/mol) for the groups of cells is markedly higher then the Redfield ratio of 6.6 for C : N. For marine heterotrophic bacteria a C : N ratio of 4.3 has been reported (Fagerbakke et al. 1996), though values ranging between 2.9 and 14.3 have been reported for bacteria generally (*see* Fagerbakke et al. 1996 and references therein). Vrede et al. (2002) found C : N values of 3.8 to 6.3 in exponentially growing cells from four marine heterotrophic bacteria, compared with a narrow range of between 3.6 and 4.1 found with the same strains grown under C limitation.

Bertilsson et al. (2003) have compared the C : N : P ratios of *Synechococcus* WH 8012 and *Synechococcus* WH 8103 grown at Redfield (N : P = 16) and P limitation (N : P = 800). For both strains an increase in C : N ratios (mol/mol) from 5.0–5.7 during exponential growth to 7.2–7.5 at P limitation was found, while the molar C : P ratios increased from 130–165 at exponential growth to 700–780 at P limitation. Also, the N : P ratios (24–33 during logarithmic growth)

Table 6. Estimated concentrations of sodium, magnesium, chlorine, potassium, and calcium in cells of *Synechococcus* strains WH 7803 and WH 8103. The number of cells analyzed is the same as for Table 4.

Strain/growth condition	$[Na^+]$*	$[K^+]$	$[Mg^{++}]$	$[Ca^{++}]$	$[Cl^-]$
WH 7803, 10 mmol L^{-1} Mg^{++} ASW	149±9†	45±3	83±6	90±10	92±6
WH 8103, 10 mmol L^{-1} Mg^{++} ASW	480±20	59±2	70±4	73±7	107±5
WH 7803 ASW	200±10	63±4	103±7	28±2	102±5
WH 8103 ASW	250±10	112±5	138±6	31±3	156±5
WH 7803 PCRS-11	220±20	130±10	118±7	50±6	150±10
WH 8103 PCRS-11	90±10	60±10	110±20	24±6	110±20

* mmol L^{-1}.

† SE.

point in the direction of a relatively low P content of these cells, and thus the strains studied by Bertilsson et al. (2003) show clear similarities to the results we obtained for WH 8103 here. Our results, though, indicate a marked difference in C : N : P stoichiometry between the two strains of *Synechococcus* WH 8103 and WH 7803 under study here. Further, with clearly visible polyphosphate formation observed for strain WH 7803, there is the suggestion of strain-specific differences in P-storage capacity among representatives of this genus.

Studies of the C : N : P ratio of diazotrophic cyanobacteria from the Baltic Sea showed low molar ratios for C : N (4.3) and C : P (32) in the spring and a gradual increase during the growth season, reaching ratios of 6.5 and 420 (mol/mol), respectively, in late August and early September (Larsson et al. 2001). These results indicate the ability of some cyanobacteria to store phosphorus and thus demonstrate the great plasticity in elemental ratios among this group of planktonic microorganisms.

For WH 8103, a bimodal distribution of C content is observed (Fig. 2). This bimodal distribution is also seen for other parameters such as N, O, P, volume, and the content of ions (Fig. 4). If we treat the two distributions separately, they are significantly different for most parameters except the C : N ratio, which is 10 for both groups. In general there is a threefold to fivefold reduction in elements between the ''high'' and ''low'' density groups, and the most striking difference was the relatively higher P content in the low density group compared to the high density group. A variability in C : vol of 0.04 to 0.36 was found for cells in the whole sample. We compared the gray level values (electron density) for these cells (*see* Löferer-Krößbacher et al. 1998) to the parameters measured but found no significant correlation to any other variable. We believe that these differences are related to cell physiology and that the results demonstrate the great variability in physiological condition of cells even when grown in monoculture.

The ion content of *Synechococcus* was dominated by Na^+ and Mg^{++} without any systematic differences between the strains. For WH 8103 the Na^+ content varied from 90 mmol L^{-1} (PCRS-11) to 480 mmol L^{-1} (10 mmol L^{-1} Mg^{++} ASW), but these differences were not reflected by any identified changes in other ions. For Mg^{++} a relatively low and invariable content of Mg^{++} was found, 70–138 mmol L^{-1}. For cells grown at a reduced (10 mmol L^{-1}) level of Mg^{++} in the growth medium, we found an increased level of Ca^{++} in both strains. Magnesium is important for various biological processes, including enzyme activity and complex formation; stabilization of proteins, e.g., polyribosomes, but at high (10 mmol L^{-1}) concentrations (Spirin 1990); and tRNA binding properties (up to 20 mmol L^{-1} Mg^{++} required) (Robertson and Wintermeyer 1987). For *Escherichia coli* it has been reported that about 100 mmol L^{-1} Mg^{++} is bound to the macromolecule fraction (Moncany and Kellenberger 1981). Since we find Mg^{++} content in the range of 70–138 mmol L^{-1} in *Synechococcus*, this is about the amount that should be bound in the macromolecular fraction of the cell. By lowering the external Mg^{++} concentration to 10 mmol L^{-1}, we observed for both strains an increase in Ca^{++} content. If we calculate the sum of divalent cations (Table 6), concentrations in the range of 131 to 173 mmol L^{-1} were obtained. The exception to this general trend is the low density fraction of WH 8103 PCRS-11, which has 45 and 6 mmol L^{-1} of Mg^{++} and Ca^{++}, respectively. For marine heterotrophic bacteria, a variable cellular content of Mg^{++} has been observed, in the range of 130 to 700 mmol L^{-1}, while Ca^{++} content was similar to that seen for *Synechococcus* here (Fagerbakke et al. 1999). We conclude that Mg^{++} in *Synechococcus* is not part of the cell's osmoregulatory system and that Mg^{++} and Ca^{++} reflect the macromolecule content of the cells, even though these ions partly could be bound to polyphosphate in strain WH 7803.

In Fig. 9, a plot from canonical variate analysis summarizes the total dispersion of the strains/growth conditions included for the *Synechococcus* samples. The two axes shown in Fig. 9 explain near 93% of the total dispersion. Axis 1 (80%) reflects mainly the contrast between Cl^-, K^+, Mg^{++}, and Na^+, C, S, while axis 2 (13%) is based on K^+, C, Mg^{++}, Na^+ versus Cl^-, N, P, and S. It is interesting that a reduction of Mg^{++} from 50 to 10 mmol L^{-1} so clearly influences the distribution of these groups. It would thus appear that it is the growth conditions used, rather than between-strain differences, which define the elemental composition of these organisms.

Biogeochemical implications—The potential ecological significance of the results we report here is clearly dependent on the extent we can extrapolate our culture study data to the natural environment. Certainly, high N : P values (i.e., $>$20) have been reported in oligotrophic regions of the equatorial Atlantic and Panama Basin (Bishop et al. 1977, 1980), areas where picocyanobacteria can dominate, which is consistent with the values for *Prochlorococcus* and *Synechococcus* we report here.

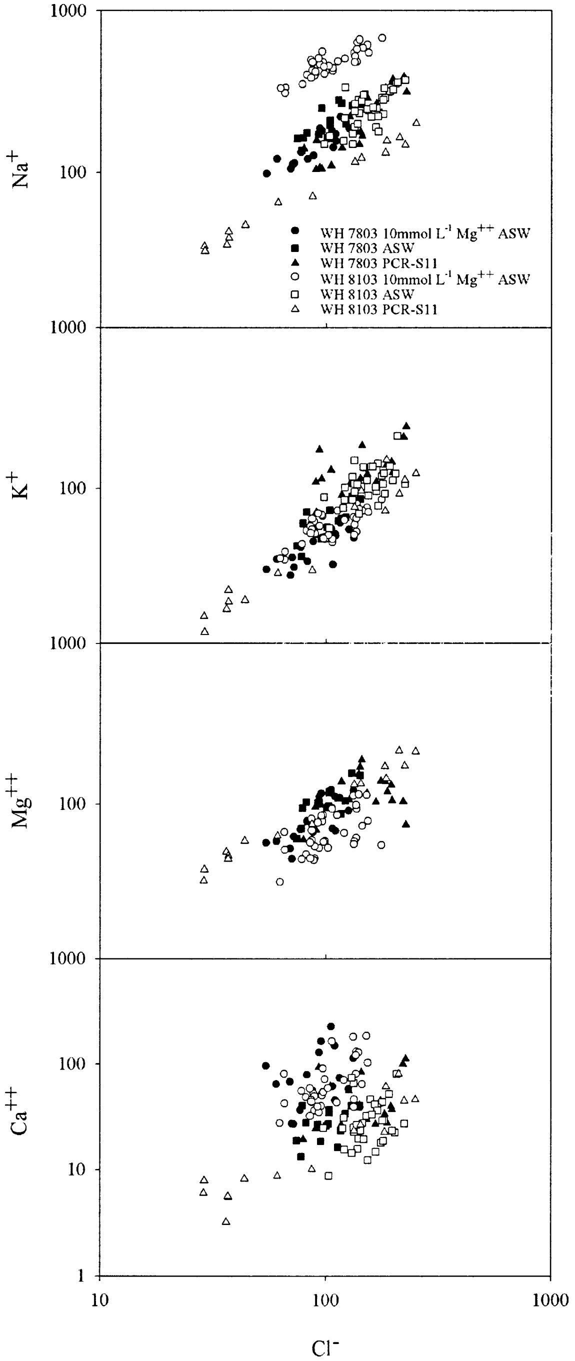

Fig. 7. Estimated cellular content of diffusible ions, Na^+, K^+, Mg^{2+}, and Ca^{2+} plotted versus Cl^- for the analyzed *Synechococcus* cells.

Thus, since *Prochlorococcus* and *Synechococcus* dominate the phytoplankton in the open ocean and subtropical gyres and since open ocean environments cover a large surface area, then C : N : P ratios in these organisms, and hence the oligotrophic environments these organisms represent, might be considered quite different from the average Redfield ratio values generally reported. This has implications for what is interpreted from Redfield ratios (e.g., export production, nutrient-based productivity calculations from C : N

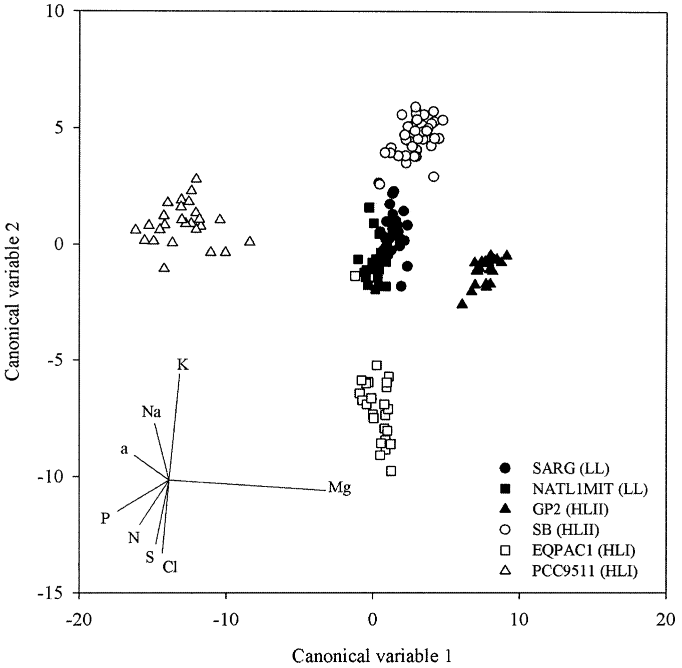

Fig. 8. Bivariate plot of the first two axes in the canonical variate analysis of the size and elemental content of six strains of *Prochlorococcus*. The length and directions of the vectors indicate the relative contribution from major parameters to the total dispersion of the populations.

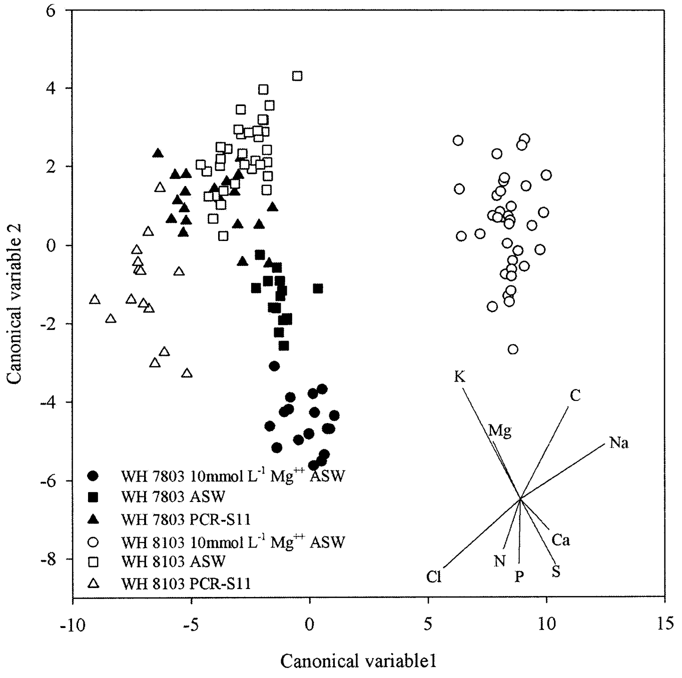

Fig. 9. Bivariate plot of the first two axes in the canonical variate analysis of the size and elemental content of *Synechococcus* strains WH 7803 and WH 8103 grown in different media. The length and directions of the vectors indicate the relative contribution from major parameters to the total dispersion of the populations.

ratios, or inferring N or P limitation from N:P ratios; *see* Geider and LaRoche 2002). On a different point, although we know little of the critical N:P ratio in marine cyanobacteria (i.e., the point where growth rate is colimited by the two nutrients and that marks the transition between N and P limitation), even given the low P content of these cells the inability to store P may lend *Prochlorococcus* more susceptible to P limitation while *Synechococcus* (with the high N requirement of the phycobilisome) may be more susceptible to N limitation.

The high C:P ratios found in all *Prochlorococcus* and in some *Synechococcus* strains may be related to their ecological role as dominating phytoplankters in oligotrophic areas. Many of the marine oligotrophic areas appear, at least intermittently, to be P limited (Thingstad and Rassoulzadegan 1995; Cotner et al. 1997; Karl et al. 1997; Wu et al. 2000), and competition for phosphate therefore presumably is severe. A low relative P requirement might therefore seem advantageous. To get a more quantitative evaluation of this, one may consider the specific affinity, which represents the volume of water cleared for phosphate per biomass per hour. For a spherical cell, a theoretical expression for maximum specific affinity $\alpha = 3D/\sigma r^2$ can be estimated (e.g., Thingstad and Rassoulzadegan 1999) based on the assumption of diffusion limited growth, i.e., that all molecules transported by diffusion to the cell surface are captured. Here D is the diffusion constant for phosphate, σ is the internal phosphate concentration, and r is the cell radius. The effect of the small r of picoplanktonic species such as *Synechococcus* and *Prochlorococcus* can thus in principle be further enhanced by a low σ. From our data, the *Prochlorococcus* strains investigated have a lower mean σ (2.9 pg P μm^{-3}) than *Synechococcus* (3.9 pg P μm^{-3}), though this difference in σ between the two species in our data set is not statistically significant (t-test, $p = 0.11$). Insertion of mean values for r and σ computed from the data in Tables 1 and 4 gives α values of 1.2 ± 0.3 and 0.27 ± 0.10 L nmol P^{-1} h^{-1} for *Prochlorococcus* and *Synechococcus*, respectively. The value estimated for *Synechococcus* is remarkably consistent with the maximum estimate of Moutin et al. (2002) of ca. 0.25 L nmol P^{-1} h^{-1} based on uptake of $^{33}PO_4$ in natural surface communities from the eastern Mediterranean. Our results do not, however, explain why Mediterranean surface waters are dominated by *Synechococcus* and not by *Prochlorococcus* (Moutin et al. 2002), as would be expected considering these affinity values alone. The expression for phosphate affinity under diffusion limitation can be rearranged to $\alpha = 4\pi Dr/p$, where p is P content per cell. A cell that can increase r by increasing its C content, without a proportional increase in p, may thus gain a competitive advantage under nutrient-limited conditions. The argument sometimes met that small-celled species have a competitive advantage due to their high surface:volume ratio is thus not true if a volume increase can be obtained without a concomitant expense in the form of increased requirement for the limiting nutrient. From such a perspective, these photosynthetic bacteria may be speculated to share the strategy of ''appearing larger than they are'' previously suggested for diatoms (Thingstad 1998), although for diatoms the volume increase has been proposed to be obtained through a nutrient-free vacuole made possible when rigidity is maintained by a Si exoskeleton. A high C content of *Synechococcus* and *Prochlorococcus* may thus provide more ecological advantages than just serving as C storage for potential future energy-limited situations.

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Received: 10 October 2002
Accepted: 24 February 2003
Amended: 26 March 2003