

## ORIGINAL ARTICLE

# Mg<sup>2+</sup> as an indicator of nutritional status in marine bacteria

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**Cells maintain an osmotic pressure essential for growth and division, using organic compatible solutes and inorganic ions. Mg<sup>2+</sup>, which is the most abundant divalent cation in living cells, has not been considered an osmotically important solute. Here we show that under carbon limitation or dormancy native marine bacterial communities have a high cellular concentration of Mg<sup>2+</sup> (370–940 mM) and a low cellular concentration of Na<sup>+</sup> (50–170 mM). With input of organic carbon, the average cellular concentration of Mg<sup>2+</sup> decreased 6–12-fold, whereas that of Na<sup>+</sup> increased ca 3–4-fold. The concentration of chlorine, which was in the range of 330–1200 mM, and was the only inorganic counterion of quantitative significance, balanced and followed changes in the concentration of Mg<sup>2+</sup> + Na<sup>+</sup>. In an osmotically stable environment, like seawater, any major shift in bacterial osmolyte composition should be related to shifts in growth conditions, and replacing organic compatible solutes with inorganic solutes is presumably a favorable strategy when growing in carbon-limited condition. A high concentration of Mg<sup>2+</sup> in cells may also serve to protect and stabilize macromolecules during periods of non-growth and dormancy. Our results suggest that Mg<sup>2+</sup> has a major role as osmolyte in marine bacteria, and that the [Mg<sup>2+</sup>]/[Na<sup>+</sup>] ratio is related to its physiological condition and nutritional status. Bacterial degradation is a main sink for dissolved organic carbon in the ocean, and understanding the mechanisms limiting bacterial activity is therefore essential for understanding the oceanic C-cycle. The [Mg<sup>2+</sup>]/[Na<sup>+</sup>]-ratio in cells may provide a physiological proxy for the transitions between C-limited and mineral nutrient-limited bacterial growth in the ocean's surface layer.**

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## Introduction

Bacteria maintain a cellular osmotic pressure essential for growth and cell division (Csonka, 1989; Martin *et al.*, 1999), and both bacteria and archaea have developed strategies for osmoadaptation under various salinity and ionic conditions (Roessler and Müller, 2001). Among these strategies is the use of compatible solutes, such as zwitterionic organic solutes (for example, proline and glycine betaine) or non-ionic compounds in bacteria (Dinnbier *et al.*, 1988; Csonka, 1989). Most of the archaea studied have been reported to have a high intracellular concentration of inorganic ions, and under optimal growth conditions K<sup>+</sup> is dominating (Martin *et al.*, 1999). Archaea is also reported to contain proteins that are rich in acidic amino acids, resulting in a net negative charge in the cells (Roessler and Müller,

2001 and references therein). The various strategies of osmoadaptation have been suggested to maintain equilibrium between macromolecule surfaces and the water phase through regulation of intracellular water density (Martin *et al.*, 1999). In addition, various solutes add to the protection and stabilization of macromolecules in the cells. K<sup>+</sup> has been shown to be an important transient osmolyte in both bacteria and archaea cells during osmotic changes, when cells switch to organic osmolytes (Dinnbier *et al.*, 1988). As there is a coupling between organic compatible solutes and osmoregulation in bacteria, one would expect carbon availability to at least in part influence the ionic content of the cells.

Heterotrophic marine bacteria may in general be limited by the availability of dissolved organic carbon or inorganic nutrients. In the ocean's interior, where the concentration of inorganic nutrients is relatively high, bacterial growth is presumably limited by the scarcity of degradable organic compounds. However, in the photic zone bacteria may be limited by the availability of dissolved organic carbon or of inorganic nutrients, for example, N, P or Fe (Tortell *et al.*, 1996; Kirchman and

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Rich, 1997; Rivkin and Anderson, 1997; Thingstad *et al.*, 1998; Van Wambeke *et al.*, 2002), a situation that allows accumulation of otherwise degradable organic compounds (Williams, 1995; Thingstad *et al.*, 1997). There are also regional differences as bacteria in some oceanic regions seem to be limited by the availability of organic carbon, whereas in others inorganic nutrient limitation seems to prevail (Rivkin and Anderson, 1997; Van Wambeke *et al.*, 2002). In addition, the metabolic balance of the open sea is also variable, with short-lived and aperiodic bursts in net primary production (Karl *et al.*, 2003), suggesting that carbon and inorganic nutrient limitation are time-variable growth conditions for the heterotrophic bacteria.

There are a few, if any, cellular signatures reported that characterize carbon or energy deprivation in native marine bacteria. Given the stable osmolarity of seawater, any major shift in osmolyte composition of marine bacteria should be related to the growth conditions of the cells, and we hypothesize that the osmolyte composition may be used as a probe for their physiological status and possibly as an indicator of dormancy (Lennon and Jones, 2011). To investigate this hypothesis we followed the elemental composition of bacteria during a phytoplankton spring bloom when the ecosystem shifts from a famine winter situation to a productive and nutrient-rich spring situation, and during shift-up experiments where glucose was added as a carbon source to presumably carbon-limited natural bacterial communities. For quantitative elemental analysis of single cells we used a transmission electron microscope equipped for dispersive X-ray micro analysis (Heldal *et al.*, 1985; Norland *et al.*, 1995).

## Materials and methods

### Field study

Samples were collected at 5 m depth in Raunefjorden using a Ruthner sampler and brought to the laboratory within 2 h. Raunefjorden is located on the west coast of Norway about 30 km south of Bergen, and the sampling period covered was January–May 2003.

### Carbon shift-up experiment

The seawater samples used in these experiments were collected as described for the field study on 7 May 2003 and 2 March 2004, when the heterotrophic bacterial community appeared carbon-limited (see Discussion). To test the physiological response of the bacterial communities to increased carbon supply, we added glucose (3 µM final concentration) to 1-l samples and incubated them for 24 h in the dark at 6 °C. Untreated 1-l samples were incubated in parallel as control. Samples for transmission electron microscope-X-ray micro analysis and denaturing gradient gel electrophoresis (DGGE) were collected at start, and were analyzed after 24 h as described below.

### Chlorophyll

Chlorophyll fluorescence was measured *in situ* using a chlorophyll fluorometer (Seapoint Sensors Inc., Exeter, NH, USA) and converted to mg m<sup>-3</sup> Chl *a* using a predetermined conversion factor of 1.4.

### Flow-cytometer counting of bacteria

All flow-cytometer analyses were performed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), equipped with an air-cooled laser providing 15 mW at 488 nm and with standard filter set-up. Enumeration of bacteria was performed on samples fixed with glutaraldehyde (2% final concentration) diluted 10- to 100-fold, and stained with SYBR Green I as described earlier (Marie *et al.*, 1999; Larsen *et al.*, 2001).

### Preparation of cells for transmission electron microscope—X-ray

Water samples were filtered through 5-µm nucleopore filters to remove larger particles and the particulate fraction in the filtrate was then concentrated 10 times by centrifugation (3400 g for 45 min), decantation and resuspension of the pellet in 1/10 of the supernatant. The concentrated particles were harvested directly onto electron microscope grids by centrifugation at 15 000 g for 10 min and 20 °C in a Beckman L8-70M ultracentrifuge (Beckman Coulter Inc., Fullerton, CA, USA) using a SW41 Ti swing-out rotor and air dried (Heldal *et al.*, 1985). The grids we used were 100-mesh Al grids (Agar Scientific Ltd, Stansted, UK) supported with a carbon-coated formvar film.

### Elemental analyses

Cells were viewed and analyzed for elements under a Philips CM 200 electron microscope (Europe Nano-Port, Eindhoven, The Netherlands). The microscope was operated in scanning mode at a tilt angle of 38°, 80 kV acceleration voltage, magnification 7800 ×, spot size 14 nm (spot size 3) and an accumulation time (live time) of 30 s. X-ray spectra were recorded from individual cells and from similar areas of the formvar film for background subtraction (Norland *et al.*, 1995). The element detection system consisted of EDAX detector DX-4 (EDAX Inc., Mahwah, NJ, USA) supported with SIS Soft Imaging Software (Olympus Soft Imaging Solutions GmbH, Münster, Germany). Carbon measurements were calibrated using latex beads of known size (Agar Scientific Ltd), and calibration constants for the different elements were determined as described elsewhere (Heldal *et al.*, 1985; Norland *et al.*, 1995).

### PCR-denaturing gradient gel electrophoresis (DGGE)

PCR-DGGE analysis was performed as described in Sandaa *et al.* (2003). In short, 20 ml of water samples were filtered on 0.2-µm DynaGard hollow-fibre syringe filters (Microgon Inc., Laguna Hills, CA,

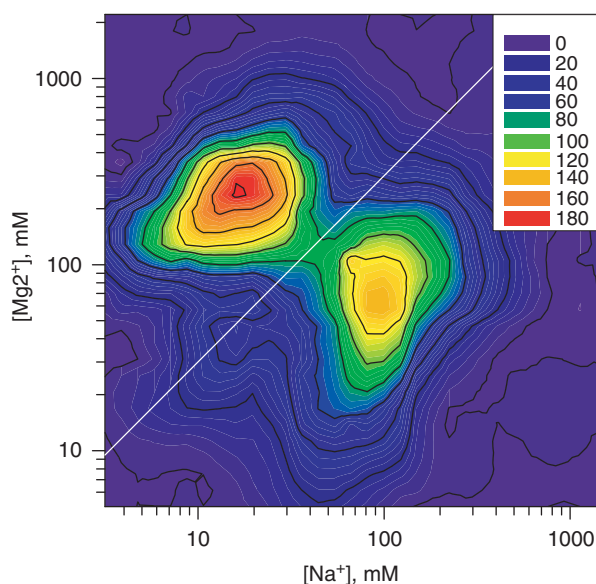
USA), and stored at  $-70^{\circ}\text{C}$  until further processing. DNA was extracted from the filters using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), and PCR amplified using the primer combinations EUBf (Giovannoni *et al.*, 1990), with a GC-clamp, and PRU517r (Lane *et al.*, 1985), with PCR conditions according to Sandaa *et al.* (2003). DGGE was performed with a Dcode 16/16-cm gel system (Bio-Rad, Herts, UK), and the gels were stained with SYBR Green II (Molecular Probes, OR, USA) before they were photographed.

## Results

By assembling data from some of our earlier investigations (Fagerbakke *et al.* (1999) and different unpublished datasets), we find that bacteria in marine ecosystems may be divided into two physiologically different populations according to their elemental composition: those with  $[\text{Mg}^{2+}]/[\text{Na}^{+}] > 3$  (molar ratio), showing signs of carbon limitation with less carbon per cell and less carbon cell quotas (C/P and C/N), and those with  $[\text{Mg}^{2+}]/[\text{Na}^{+}] < 3$ , with more carbon per cell and higher carbon cell quotas (Figure 1, Table 1, Supplementary Table 1).

### Field study

The main diatom spring bloom occurred in March and peaked with a chlorophyll concentration of about  $5\text{ mg Chl } a\text{ l}^{-1}$  (Figure 2a). Total counts of bacteria (Figure 2b) showed an increase during the early stage of the bloom, while the major increase in bacterial biomass followed the collapse of the bloom (late March to early April).



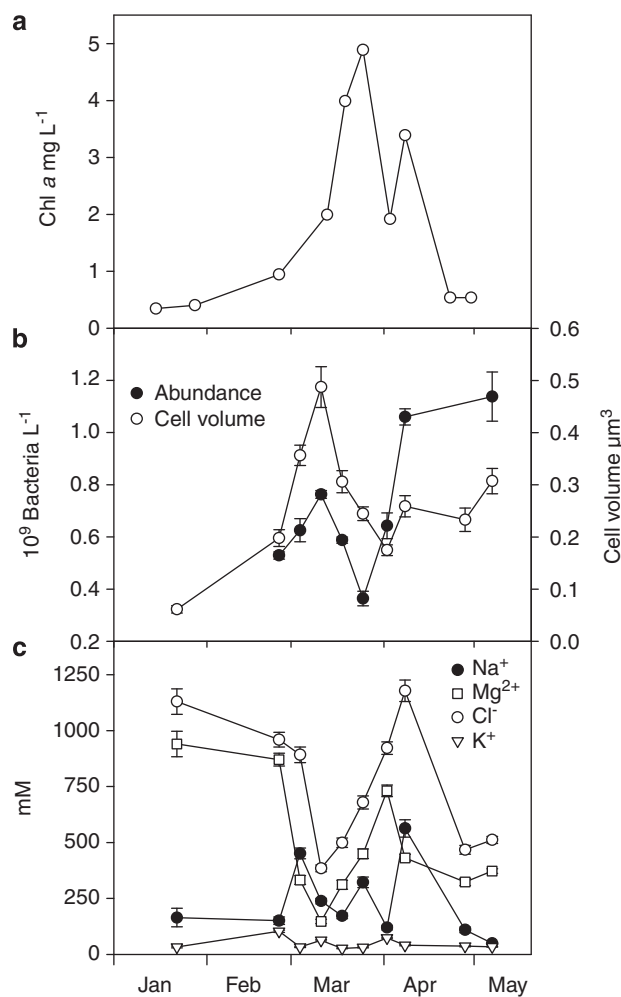
**Figure 1** Contour plot of the cellular concentrations of  $\text{Mg}^{2+}$  and  $\text{Na}^{+}$  in 2549 single native bacteria from various marine environments. Colours indicate number of cells per bin in the  $10 \times 10$ -bin matrix used for plotting. The white line shows the molar ratio  $[\text{Mg}^{2+}]/[\text{Na}^{+}] = 3$ . Additional data on the cells with high ( $>3$ ) and low ( $<3$ ) cellular  $[\text{Mg}^{2+}]/[\text{Na}^{+}]$  are given in Table 1 and in Supplementary Table 1.

X-ray analysis of single cells revealed that  $\text{Mg}^{2+}$  and  $\text{Na}^{+}$  were the major cations, and that  $\text{Cl}^{-}$  was the dominating anion (Figure 2c). The  $\text{Na}^{+}/\text{Cl}^{-}$  molar ratio in the cells varied from 0.1 to 0.6, implying that salts in the seawater, where this ratio is constant at 0.85, did not affect our measurements (cf Figure 3). The changes in total concentrations and molar ratios of inorganic ions correspond to the onset of the algal bloom and changes in algal biomass (Figure 2a). In January and February, when the phytoplankton biomass was low, the bacteria

**Table 1** Carbon content, C/N and C/P ratio measured in 2549 single bacteria with high ( $>3$ ) and low ( $<3$ ) cellular  $[\text{Mg}^{2+}]/[\text{Na}^{+}]$

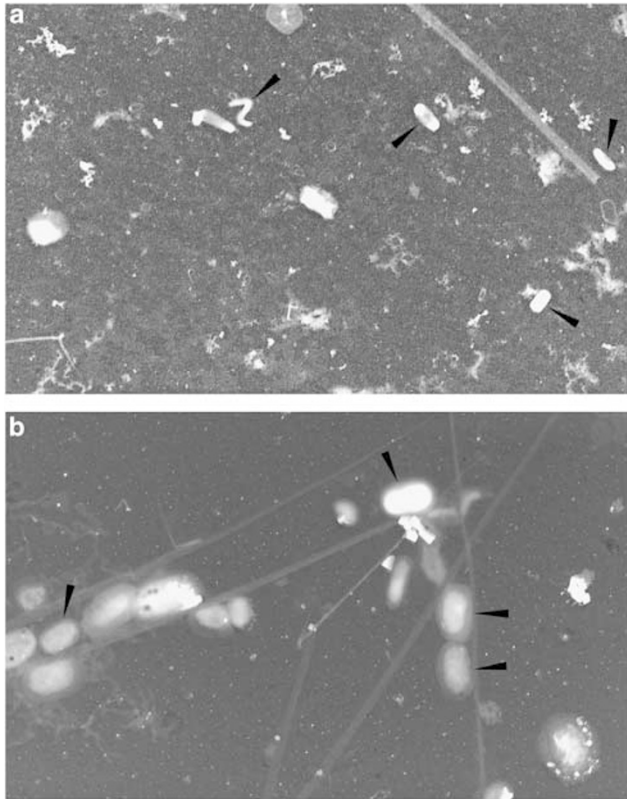
$[\text{Mg}^{2+}]/[\text{Na}^{+}]$ (mol/mol)	C/vol. (fgC/ $\mu\text{m}^3$ )	C/cell (fg)	C/N (mol/mol)	C/P (mol/mol)	N
$>3$	$96 \pm 2$	$16.3 \pm 0.5$	$5.11 \pm 0.04$	$68 \pm 2$	1167
$<3$	$85 \pm 1$	$26.3 \pm 0.8$	$6.31 \pm 0.05$	$99 \pm 3$	1382

Values are mean  $\pm$  s.e. Additional data are given in Supplementary Table S1.



**Figure 2** Seasonal variation of (a) Chl *a*, (b) bacterial total counts and cell volume, and of (c) ion content in bacteria from Raunefjorden during winter and spring 2003.

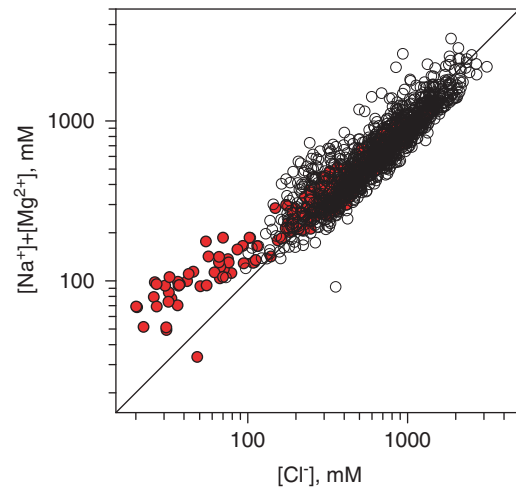




**Figure 3** Transmission electron microscope images of unfixed and unstained cells (arrow heads) used for quantitative analysis of elements (single-cell TEM-XRMA). The samples are from 22 January (a) and 18 March (b) 2003, and the images illustrate the shift in bacterial cell size during the phytoplankton spring bloom when carbon availability increases and the molar  $[Mg^{2+}]/[Na^+]$  ratio in the cells shifts from high to low. They also illustrate that there is no visible precipitation of salt in or around the cells that could affect the elemental analysis.

had high  $Mg^{2+}$  levels (870–940 mM), low  $Na^+$  concentration (150–160 mM) (Figure 2c) and the cells were small (Figures 2b and 3a). In early March, at the onset of the spring bloom, the cells also grew in size (Figures 2b and 3b), and there was a marked reduction in cellular  $Mg^{2+}$  concentration (150–330 mM) accompanied by an increase in  $Na^+$  concentration (240–450 mM) (Figure 2c). The decrease in phytoplankton biomass in late March and early April was accompanied by a major increase in bacterial biomass, and an increase in the bacterial content of  $Mg^{2+}$  and  $Cl^-$  before the contents of these ions again decreased in late April and early May (Figure 2c). Notably, the seasonal change in cellular  $K^+$  concentration was small in absolute terms (27–103 mM), and this ion thus appears to be of minor importance with respect to osmotic regulation in marine bacteria (Figure 2b) during normal growth.

For cells with higher concentrations of  $Mg^{2+}$  and  $Na^+$ , there was a strong tendency towards a 1:1 relationship between the concentrations of  $Mg^{2+} + Na^+$  and the concentration of  $Cl^-$  in individual cells (Figure 4). But in the beginning of March when the total cellular concentration

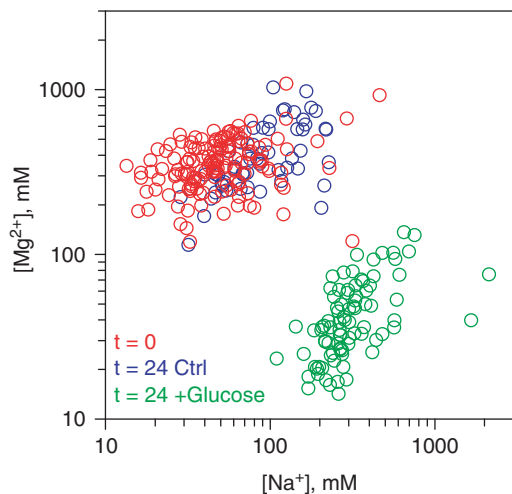


**Figure 4** Single-cell estimates of  $[Na^+] + [Mg^{2+}]$  versus  $[Cl^-]$  for the 1550 cells analysed in the time series from Raunefjorden. The red data points mark the cells sampled on 11 March, when there was a deficiency of inorganic anions ( $Cl^-$ ) compared with cations ( $Mg^{2+} + Na^+$ ). The line shows the 1:1 ratio.

of inorganic ions decreased to  $0.850 \text{ Osmol l}^{-1}$ , which is lower than the osmolarity of seawater ( $1.0\text{--}1.1 \text{ Osmol l}^{-1}$ ), there is a deficiency of inorganic anions ( $Cl^-$ ) compared with cations ( $Mg^{2+} + Na^+$ ). It is thus reasonable to speculate that the cells in this situation used negatively charged organic compatible solutes as part of their osmotic potential.

#### Carbon shift-up experiment

In samples collected in Raunefjorden during early May 2003 we measured an average cellular concentration of  $370 \pm 11 \text{ mM } Mg^{2+}$  and  $49 \pm 4 \text{ mM } Na^+$  (mean  $\pm$  s.e.,  $n = 178$ ) (Figure 2c, Supplementary Table 2). When these samples were incubated for 24 h with glucose as carbon source the cellular concentration of  $Mg^{2+}$  decreased to  $35 \pm 3 \text{ mM}$ , whereas the  $Na^+$  concentration increased to  $350 \pm 25 \text{ mM}$  ( $n = 111$ ) and became the dominating cation (Figure 5 Supplementary Table 2). The changes observed after 24 h in control cells were small (Figure 5). The inorganic osmolarity ( $[Mg^{2+}] + [Na^+] + [Cl^-]$ ) of the cells decreased from  $0.9\text{--}1.2$  to  $0.7 \text{ Osmol l}^{-1}$  in the glucose-amended cells whereas in the control cells the inorganic osmolarity increased to  $1.1\text{--}1.5 \text{ Osmol l}^{-1}$ . Moreover, the abundance of bacteria increased significantly more in the glucose-amended cultures during the incubation than in the control culture (Supplementary Table 2,  $P < 0.001$ ,  $t$ -test), suggesting that bacterial growth was stimulated by the addition of carbon, and thus that the cells were carbon-limited at the start of the experiment. A second experiment carried out 1 year later gave similar results (Supplementary Table 2). Thus, adding glucose to cells that were carbon-limited seems to mimic the effect seen for the cellular ion concentrations before and after the spring bloom.



**Figure 5** Single-cell estimates of  $[\text{Mg}^{2+}]$  versus  $[\text{Na}^+]$  for the 350 cells analysed in the carbon addition experiment (cf. Supplementary Table S1).

To test whether this apparently universal change in osmolyte composition could be explained as a total shift in community composition during the 24-h incubation, we analysed samples collected at 0 and 24 h by PCR-DGGE (Supplementary Figure 1). The numbers of bands were the same at 0 and 24 h, as six bands were common whereas six bands were unique to each of the two samples. Thus, the change in osmolyte composition cannot be explained as a total shift in community composition, and we conclude conservatively that all the 18 different populations that could be detected as a visible band on the DGGE gel behaved in a similar way, in that they had either a high  $[\text{Mg}^{2+}]/[\text{Na}^+]$ -ratio at C-limitation (6 populations), a low  $[\text{Mg}^{2+}]/[\text{Na}^+]$ -ratio at C-surplus (6 populations) or both (6 populations).

## Discussion

The basic interpretation of the carbon shift-up incubation experiment is that there is an inverse relationship between carbon availability and cellular  $[\text{Mg}^{2+}]/[\text{Na}^+]$ -ratios in bacteria in which C-limited cells have a high cellular  $[\text{Mg}^{2+}]/[\text{Na}^+]$ -ratio, whereas C-sufficient cells have a low  $[\text{Mg}^{2+}]/[\text{Na}^+]$ -ratio. The diatom-dominated phytoplankton spring bloom represents an important net input of organic carbon to the ecosystem, and this input prompts a response in the physiological state of the bacteria (Figure 2c). Starting from a pre-bloom situation with bacteria that have a high  $[\text{Mg}^{2+}]/[\text{Na}^+]$  ratio and are C-limited, the situation shifts to C-sufficiency and a low  $[\text{Mg}^{2+}]/[\text{Na}^+]$  ratio during the bloom. This situation is then followed less intuitively by a new period with high  $[\text{Mg}^{2+}]/[\text{Na}^+]$  ratio, which we interpret to indicate C-limitation. Models allowing for both mineral- and carbon-limited growth (Thingstad *et al.*, 1998) predict

increased probability for C-limitation in cases when the biomass is shifted towards phytoplankton predators characterized by a low biomass of mineral nutrient competitors and a high rate of recycling. The oscillatory pattern in bacterial physiology may thus reflect predator-prey oscillations in the microbial food web. The low C/N and C/P ratios of 5–7 and 40–65 (mol/mol), respectively, estimated from our X-ray measurements (data not shown) indicate that the bacteria were not severely nutrient (N or P) limited throughout the sampling period. Tuomi *et al.* (1995) reported transient shifts in the cellular  $[\text{Mg}^{2+}]/[\text{Na}^+]$  ratio of marine bacteria in response to both carbon and phosphate addition. Caution is called when interpreting this limited data set, but the study, nevertheless, suggests that shifts in cellular  $[\text{Mg}^{2+}]/[\text{Na}^+]$  ratio may not exclusively be related to availability of carbon.

Our analyses show that the concentrations of  $\text{Mg}^{2+} + \text{Na}^+$  are approximately at the same level as  $\text{Cl}^-$  concentration of the cells (Figure 4). A similar balance of  $\text{Mg}^{2+} + \text{Na}^+$  vs  $\text{Cl}^-$  has been reported by Tuomi *et al.* (1995). As  $\text{Mg}^{2+}$  is a divalent cation, this leads to an excess of positive charge in the cells. This could partly be compensated for by the reduced  $\text{Mg}^{2+}$  activity at high cellular ion content. As yet we have no indication of inorganic ions that could act as a counterbalance for  $\text{Mg}^{2+}$  levels shown here. However, there is an increased level of sulphur in cells with high  $\text{Mg}^{2+}$  (Supplementary Table 1), and further studies will reveal if  $\text{SO}_4^{2-}$  may contribute to the charge balance. A high content of proteins that are rich in acidic amino acids resulting in a net negative charge in the cells could also be part of the charge counterbalance. During the spring bloom we observed a reduction in total content of ions in the cells (Figure 2c). As the osmolarity in such periods gets lower than that of seawater, it is reasonable to speculate that cells use organic compatible solutes as part of their osmotic potential. The question still remains whether organic compatible solutes also represent a carbon and energy store of the cells. Gouffi and Blanco (2000) found that a range of disaccharides acted as osmoprotectants for *Sinorhizobium melioli*, but these compounds were catabolized and replaced with other endogenously synthesized osmolytes during the growth cycle. Although experimental data are scarce, it is reasonable to believe that osmoregulation in native marine bacteria is a dynamic process, where the balance between organic and inorganic osmolytes shifts as nutrient availability changes.

The concentrations of elements in the cells were estimated using total cell volume and are hence not to be considered as the concentration of free ions in the free cellular water space or osmotic volume of the cells. The volume occupied by the dry matter in the cells, ionic strength, and binding of ions to organic components and macromolecules will have to be considered to estimate the concentration of free ions in the cells. We are at present not aware of

any method that could give analytical support for such calculations.

Mg<sup>2+</sup> is the most abundant divalent cation in living cells and influences a variety of cellular processes. By binding to substrates, enzymes and cell constituents (for example, ribosomal subunits and membranes) it stabilizes these structures, and by interacting with enzyme–substrate complexes, serving as a cofactor with ATP, and influencing conformational changes of enzymes it affects enzyme activity (Stendig-Lindberg, 2001). Despite its important role in the physiology of bacteria, little is known about Mg<sup>2+</sup> transport and homeostasis (Smith and Maguire, 1998; Moncrief and Maguire, 1999). However, by being related to stability and activity, cellular Mg<sup>2+</sup> concentration could be a signature for dormancy of cells.

Textbooks in microbial physiology do not mention Mg<sup>2+</sup> as an osmotically important solute in bacterial cells, and there are few reports suggesting Mg<sup>2+</sup> to be involved in cellular osmoregulation in bacteria (Tuomi *et al.*, 1995; Fagerbakke *et al.*, 1999). Most experiments carried out to study osmotic properties in bacteria have been performed in batch cultures with sufficient nutrients available. Cells grown under these conditions may not be representative of cells living under oligotrophic conditions in seawater. The work presented here points at Mg<sup>2+</sup> as an important osmolyte in native marine bacteria.

Marine bacteria are moderately halophilic, requiring sodium for growth, and this Na<sup>+</sup> requirement has been suggested to define them as a group (Oh *et al.*, 1991). Some marine bacteria have been shown to possess a respiration-dependent primary sodium pump, and also to have NADH oxidase requiring Na<sup>+</sup> for maximum efficiency (Oh *et al.*, 1991). Thus, the elevated Na<sup>+</sup> levels we observed in the carbon-supplemented microbial communities may be related to the active metabolism of the cells, as well to the role of sodium as an osmolyte.

In this study we have reported a previously unknown physiological response to carbon limitation in marine bacteria. The response is detectable as an intracellular increase in concentration of Mg<sup>2+</sup> up to 940 mM, which is 4–5 times higher than the concentration found in C-sufficient cells. It is tempting to speculate that this response is linked to the need for an osmoregulation based on inorganic compounds during C-starvation. As the response appears to be a universal feature of marine bacteria, the [Mg<sup>2+</sup>]/[Na<sup>+</sup>] ratio may serve as a diagnostic tool for identifying the C-limitation status in natural samples, and accordingly might lead to a more thorough understanding of the carbon cycle in marine environments.

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