Accumulation of ambient phosphate into the periplasm of marine bacteria is proton motive force dependent

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Bacteria acquire phosphate (P₃) by maintaining a periplasmic concentration below environmental levels. We recently described an extracellular P₃ buffer which appears to counteract the gradient required for P₃ diffusion. Here, we demonstrate that various treatments to outer membrane (OM) constituents do not affect the buffered P₃ because bacteria accumulate P₃ in the periplasm, from which it can be removed hypo-osmotically. The periplasmic P₃ can be gradually imported into the cytoplasm by ATP-powered transport, however, the proton motive force (PMF) is not required to keep P₃ in the periplasm. In contrast, the accumulation of P₃ into the periplasm across the OM is PMF-dependent and can be enhanced by light energy. Because the conventional mechanism of P₃-specific transport cannot explain P₃ accumulation in the periplasm we propose that periplasmic P₃ anions pair with chemiosmotic cations of the PMF and millions of accumulated P₃ pairs could influence the periplasmic osmolarity of marine bacteria.
Phosphorus (P) is a macronutrient universally required by all organisms including bacteria. Bacteria have a number of ways to acquire P. Scavenging P from organic molecules and reducing growth dependence on P by substituting P with sulfur in their membranes\(^1\)-\(^4\) are both beneficial for bacteria living in phosphate (P\(\text{Ι}\))-depleted marine environments. However, there is compelling evidence that bacteria prefer P\(\text{Ι}\) to organic P molecules\(^5\)-\(^9\). Marine bacteria, which typically have two membranes (and therefore stain Gram negative), acquire P\(\text{Ι}\) using a diffusion gradient between the environment and the periplasm\(^10\),\(^11\). In their cells, the periplasm is separated from the environment by the outer membrane (OM), whose permeability to small, hydrophilic solutes is controlled mainly by hydrated channels—porins (Fig. 1a\(\dagger\))\(^1\). Porins select solutes on the basis of their size, shape and charge\(^12\),\(^13\). When nutrients such as P\(\text{Ι}\) become depleted, cells increase the number of porins to facilitate nutrient diffusion\(^14\).

There are the three known bacterial transport systems to import P\(\text{Ι}\), from the periplasm into the cytoplasm: a low affinity-high velocity phosphate inorganic transport (Pit) system, a low affinity-high velocity Na-dependent phosphate transport (Npt) system and a high affinity-low velocity phosphate-specific transport (Pst) system\(^15\)-\(^17\). Bacteria living in P\(\text{Ι}\)-depleted waters use only the Pst system\(^18\),\(^19\). The PstCAB transporter is ATP-powered and receives P\(\text{Ι}\) from a carrier protein, a PstS subunit, when the latter docks at its periplasmic side (Fig. 1a\(\dagger\)). Although the P\(\text{Ι}\) concentration of \(~10^{-7}\text{ mol} \text{l}^{-1}\) is required for efficient import of P\(\text{Ι}\) by PstSCAB\(^18\) is relatively high, it should not restrict P\(\text{Ι}\) diffusion into the periplasm, because the periplasmic volume of even a relatively large bacterial cell, e.g., Synechococcus...
cyanobacteria (Supplementary Table 1) with an estimated periplasmic depth\textsuperscript{21} of 10\textsuperscript{−8} m, is only about 2\times10\textsuperscript{−17} I. In such a tiny volume, the presence of only a few free Pi molecules would exceed the threshold 10\textsuperscript{−7} mol\textsuperscript{−1} Pi concentration (Fig. 1a\textsuperscript{f}).

Bacteria maximize the diffuse flux of nutrients through the OM by maintaining a steep nutrient concentration gradient between the environment and periplasm\textsuperscript{10,11}. Consequently, to allow efficient diffusion of Pi into the periplasm in Pi-depleted (10\textsuperscript{−5}–10\textsuperscript{−4} mol\textsuperscript{−1}) oceanic surface waters\textsuperscript{22}, the periplasmic Pi concentration should hypothetically be <10\textsuperscript{−9} mol\textsuperscript{−1}. This means that there should be no free Pi molecules in the periplasm, i.e., every Pi molecule entering the periplasm should be instantly bound by a PstS subunit requiring an affinity >100 times above the known PstS affinity limit. However, the PstS affinity requirement does not seem to limit the growth of ubiquitous SAR11 alphaproteobacteria and Prochlorococcus cyanobacteria—the two bacterial populations comprising \≤4% of oceanic surface bacterioplankton in the P-depleted North Atlantic subtropical gyre\textsuperscript{7}. Furthermore, the ecological success of these bacteria is probably related to the high rates of Pi uptake measured in the gyre\textsuperscript{22}. Surprisingly, measured rates of Pi acquisition by Prochlorococcus and SAR11 are lower in tropical surface waters where bacterial growth and Pi concentrations are higher\textsuperscript{22}. The counter-intuitive reduction in the Pi acquisition rate by faster growing bacteria was attributed to the presence of an intermediate buffer, in which both SAR11 and Prochlorococcus cells store Pi: the fuller the Pi buffer is, e.g., in P-replete tropical surface waters, the fewer Pi molecules a cell acquires slower from seawater to top the buffer up\textsuperscript{22}. As every Pi molecule acquired, or more precisely accumulated, by a cell first enters the buffer before being imported for assimilation, bacterial Pi uptake into the buffer and Pi import from the buffer can be decoupled. Although Prochlorococcus and SAR11 bacteria possess genes related to synthesis of polyphosphates\textsuperscript{23}, their cells are perhaps too small to store Pi as polyphosphates intracellularly and they somehow accumulate Pi extracellularly.

Our aim here is to explain how marine bacteria accumulate Pi extracellularly. We demonstrate that Pi accumulation rates by oceanic SAR11, Prochlorococcus and Synechococcus cells are extremely variable in the Pi-depleted North Atlantic. Lower rates can be stimulated by light energy but their maximal rates are insensitive to light and approach the theoretical upper limit of diffusion. To relate the accumulated Pi with cellular Pi requirements, the Pi contents of flow-sorted cells of oceanic Prochlorococcus and SAR11 were determined by microbeam synchrotron X-ray fluorescence (μ-SXRF) using flow-sorted cultured Synechococcus cells for calibration. Using pulse-chase experiments with \textsuperscript{33}Pi and \textsuperscript{32}Pi (\textsuperscript{3}P\textsuperscript{−}) radiotracers, we showed that cultured Synechococcus cells accumulate Pi in a similar way to SAR11 and Prochlorococcus cells\textsuperscript{22}. This justified the use of Synechococcus isolates for more intrusive tests (e.g., of retention of accumulated Pi), to curtail artefacts resulting from the low tolerance of oceanic SAR11 and Prochlorococcus bacteria to harsh experimental manipulations. Moreover, by working with artificial seawater (ASW), we expanded the experimental range of Pi concentrations below those encountered in natural Pi-depleted seawater. To examine how the extracellular Pi is retained, we developed a living cellular model system with the extracellular \textsuperscript{3}P-labelled buffer (Fig. 1a\textsuperscript{d}) and treated them with hydrolytic enzymes, surfactants, inhibitors, amended ASW and hypotonic solutions to locate whether the Pi buffer is on the cell surface (Fig. 1b\textsuperscript{c}), e.g., retained by a charged S-layer, as was recently proposed for archaea\textsuperscript{24}, or in the periplasm (Fig. 1c\textsuperscript{f}). We used specific metabolic inhibitors\textsuperscript{25–28} to determine whether the proton motive force (PMF) is driving extracellular Pi accumulation and retention.

Based on our field and laboratory experiments, we conclude that the PMF is essential for Pi accumulation (but not retention) in the periplasm of marine bacteria. As the PstS-driven import cannot explain Pi accumulation in the periplasm, we propose a mechanism of periplasmic ionic pairing of inwardly diffusing Pi anions with chemiosmotic cations of the membrane potential (Fig. 1a\textsuperscript{f}), which could conserve cellular energy and control periplasmic turgor.

**Results**

**Bacterial clearance rates of Pi.** Pi clearance rates (the volume of water cleared of Pi by a cell per unit of time, while the cell accumulates the cleared Pi) of oceanic SAR11, Prochlorococcus and Synechococcus cells vary by orders of magnitude in the patchily Pi-depleted North Atlantic (Supplementary Figs. 1 and 2a, and Supplementary Table 2). Living in such a patchy environment warrants the use of an extracellular buffer, where Pi is temporarily stored\textsuperscript{22}. Mostly the measured rates are lower in waters adjacent to the subtropical gyre with higher in situ Pi concentrations, whereas the rates are higher in the central gyre waters with lower Pi concentrations (18–28\textsuperscript{N}). Supplementary Fig. 2a and Supplementary Table 2). In the central gyre waters, maximal Pi clearance rates of SAR11, Prochlorococcus and Synechococcus cells (Fig. 2a) are only 30\times, 40\times and 25\times, respectively, below the theoretical maximal rate of nutrient acquisition by diffusion\textsuperscript{10,11}. Apparently, a Synechococcus cell can accumulate Pi faster than either a Prochlorococcus or a SAR11 cell. To understand why the abundance of Synechococcus in the central gyre waters is three orders of magnitude lower than the abundance of Prochlorococcus and SAR11, we need to take into account cell sizes and cellular Pi requirements of these bacteria.

Once their maximal Pi clearance rates are normalized to cellular biovolumes (Supplementary Table 1 and Fig. 2a) then a Prochlorococcus, a Synechococcus or a SAR11 cell can theoretically clear all dissolved Pi from seawater \≤2.5 \times 10\textsuperscript{4}, 2.6 \times 10\textsuperscript{4} or 1.2 \times 10\textsuperscript{5} times its cellular volume min\textsuperscript{−1}, respectively. In agreement with the surface area-to-volume concept\textsuperscript{29}, these biovolume-specific clearance rates show that a SAR11 cell is noticeably (4–5\times) more effective in uptake of Pi than either a Prochlorococcus cell or a Synechococcus cell probably owing to the 2\times higher surface area-to-volume ratio of a small, curved-rod shape SAR11 cell compared with the coccoid cyanobacteria (Supplementary Fig. 3 and Supplementary Table 1).

To account for the cellular Pi requirements of oceanic bacterial cells, we determined their cellular Pi content. The cellular Pi content of a Synechococcus cell (1.93 \times 10\textsuperscript{7} P atoms cell\textsuperscript{−1}) and hence their Pi demand is 5–7\times higher than the Pi content of a Prochlorococcus and a SAR11 cell (4.14 and 2.68 \times 10\textsuperscript{6} P atoms cell\textsuperscript{−1}, respectively, Fig. 2b and Supplementary Table 3). This implies that Synechococcus growth in Pi-depleted waters is constrained by its high Pi demand rather than by its ability to acquire Pi. It is also notable that a larger Synechococcus and a smaller Prochlorococcus have similar biovolume-specific Pi clearance rates, despite the 1.2\times higher cell surface-to-volume ratio of the latter (Supplementary Table 1). As porin-restricted diffusion constrains the Pi clearance rate, the OM of Synechococcus ought to be more permeable to P, e.g., to have more P-specific porins per µm\textsuperscript{2} of the OM surface.

The above comparisons show that despite its high Pi demand, a Synechococcus cell is a good model system for studying the process of Pi acquisition by oceanic bacteria. In our laboratory studies, we focused on the oligotrophic Synechococcus sp. strain WH8102 but also made comparisons with the mesotrophic Synechococcus strains WH8109 and WH7803\textsuperscript{30}. The 40–90\times lower Pi clearance rate of larger cells of cultured Synechococcus
compared with oceanic bacterioplankton suggests that *Synechococcus* cells grown at a $P_i$ concentration $>10^{-5}$ mol l$^{-1}$ have fewer porins than oceanic bacteria even when the $P_i$ concentration is downshifted (Fig. 2a). Such porin-restricted diffusion explains why a cultured *Synechococcus* cell cleared $P_i$ from about the same volume of water, while the $P_i$ concentration ranged over more than seven orders of magnitude. The $P_i$ clearance rate of cultured *Synechococcus* cells remained relatively constant between the three strains down to the low end of the tested $P_i$ concentrations (Fig. 2a), such that within a few hours these cells removed its labile pool was 10× smaller than observed in oceanic bacteria, the pulsed $^{33}P_i$ continued to accumulate in the labile macromolecules should be halted, because any of the 4.5–7×10$^{-9}$ mol l$^{-1}$ $^{33}P_i$-pulse that remains in the seawater would be diluted with the chase $>100$×. However, similar to that observed in oceanic bacteria, the pulsed $^{33}P_i$ continued to be assimilated into cell macromolecules at an unchanged rate, which started slowing down only after 5 h (Fig. 3a, c). Usually in pulse-chase experiments, incorporation of a pulsed molecule (e.g., amino acid or nucleoside) into bacterial macromolecules halts abruptly upon dilution with the chase, because the intermediate cellular labile pool of the pulsed molecule is small, e.g., 5% of the tracer incorporated into macromolecules. In the case of $P_i$, that labile pool was 10×–20× larger than the amount assimilated into cellular macromolecules (Fig. 3b, d at the 3 h time point).

We then used the difference in $^{*}P_i$ label between the total $P_i$ in live cells and macromolecule-bound $P_i$ to estimate the amount of $P_i$ in three *Synechococcus* strains. This showed the total $^{*}P_i$ content of tested live cells (Fig. 4a) to be consistently several times higher than the $^{*}P_i$ content of cellular macromolecules (fixed with either PFA or trichloroacetic acid (TCA), Fig. 1d,e and Supplementary Fig. 4). Furthermore, in the $^{*}P_i$ pre-labelled cells, which were washed and suspended in ASW with no $^{*}P_i$ (see Methods), the $^{*}P_i$ content in macromolecules increased with time and approached the total $^{*}P_i$ that remained stable (Fig. 4b). With no $^{*}P_i$ in the medium, the additional $^{*}P_i$ gained by the macromolecules can only be sourced from $^{*}P_i$ accumulated in the intermediate buffer. These data also show that the accumulated $^{*}P_i$ is semi-labile and can be imported into the cytoplasm to synthesize nucleic acids.

To confirm the extracellular (i.e., not in the cytoplasm) location of the labile $P_i$, we washed cells for 1–2 min with deionized water
Periplasmic location and retention of the Pi in *Synechococcus*. To identify the exact location of the accumulated Pi, and to assess whether Pi was associated with specific cell surface ligands, we examined the response of live *P*. *Synechococcus* cells to a variety of treatments, which are inapplicable to delicate oceanic bacteria (Fig. 1b, c and Supplementary Tables 5 and 6). Treatments with surfactants released no Pi (Fig. 6), suggesting that it is not associated directly with OM lipids. High concentrations of a broad-spectrum protease Proteinase K, which was shown to efficiently digest extracellular proteins in bacteria, did not release the accumulated Pi, implying no direct involvement of extracellular proteins (including the S-layer proteins of *WH8102* in Pi accumulation (Fig. 6). Release of the accumulated Pi did not happen after destabilization of the spatial conformation of lipopolysaccharides (LPS) by depletion of Ca²⁺ and Mg²⁺ di-cations (ASW-Pi-Ca and NaCl + EDTA) or by lysozyme treatment (ASW-Pi) (Fig. 6), suggesting no direct involvement of LPS in Pi accumulation. Retention of Pi by phosphorylation of extracellular macromolecules was ruled out, because alkaline phosphatase had no effect on the accumulated Pi (Fig. 6). Incubations with either acidic (pH 6) or alkaline (pH 10) ASW failed to remove the accumulated Pi (Fig. 6), demonstrating an insignificant effect of (de)protonation on the stability of the accumulated Pi.

To assess the role of PMF in retention of the accumulated Pi, we treated *P*. pre-labelled *Synechococcus* cells with the inhibitor carbonyl cyanide m-chloro-phenyl-hydrazone (CCCP) (Supplementary Table 7). Dissipation of the PMF had a minor effect on the already accumulated Pi: the amount of released Pi (Supplementary Table 7). Dissipation of the PMF had a minor effect on the already accumulated Pi: the amount of released Pi was comparable to the amount of Pi lost by washing live cells with ASW-Pi (Supplementary Fig. 4). In contrast to the above treatments, accumulated Pi was easily removed by short (1–2 min) rinses of live cells with a range of hypotonic solutions (DW). Washing live cells with DW effectively strips Pi and other ions adsorbed to the OM components. In addition, such brief hypo-osmotic shock also releases the content of the periplasm without disrupting the plasma membrane. Western blotting analysis confirmed the presence of the periplasmic Pi-binding subunits PstS in the DW-washed fraction but detected no Rubisco, arguably the most abundant soluble protein in the cyanobacterial cytoplasm (Fig. 5a, b). In fact, even a 30 min incubation of *Synechococcus* in DW did not lyse the cells (Fig. 5c). As a substantial fraction of the accumulated *Pi* was removed with a brief rinse of live *Synechococcus* with DW (Fig. 3), we therefore conclude that their cells accumulate Pi outside the plasma membrane, i.e., on the surface of the OM or in the periplasm (Fig. 1a†).
### Fig. 4 Accumulation and retention of the accumulated $^*\text{P}_i$ by *Synechococcus*

**a** The total $\text{P}_i$ accumulated in live cells (grey bars) compared with the amount of $\text{P}_i$ in PFA-fixed cellular macromolecules (red bars) and TCA-precipitated nucleic acids (blue bars). Cells washed to remove the free $\text{P}_i$ were re-suspended in ASW-$\text{P}_i$, supplemented with 100 Bq ml$^{-1}$ of H$^{32}$PO$_4$, incubated under standard cultivation conditions and sampled after 2 and 4 h.

**b** Retention of the total accumulated $\text{P}_i$ in $^*\text{P}_i$-prelabelled cells and its internalization into macromolecules and nucleic acids. The pre-labelled cells re-suspended in ASW-$\text{P}_i$ were incubated under standard cultivation conditions and sampled after 1 and 19 h. The plots show the results of representative experiments. Bars present mean values of technical duplicates indicated as crosses. Horizontal grey lines indicate major ticks of the Y-axis to assist comparing values. Source data are provided as a Source Data file.

(Fig. 7), i.e., ASW diluted 1:10–1:100, phosphate-buffered saline or DW (Supplementary Table 8), which release periplasmic cell contents$^{27,29}$ but do not cause osmotic cell lysis of marine *Synechococcus* (Fig. 5). Hypotonic washes reproducibly removed an amount of $^*\text{P}_i$ less than, or similar to, that removed from the PFA-fixed cellular macromolecules (Figs. 3 and 7). Thus, resistance to a variety of extracellular treatments (Fig. 6) in conjunction with the hypotonic removal of the accumulated $\text{P}_i$ (Figs. 3, 5 and 7) strongly indicates the periplasmic location of the accumulated $\text{P}_i$.

**The PMF is essential for $\text{P}_i$ accumulation.** The inhibitor CCCP has previously been used to investigate the role of the PMF in various aspects of cyanobacterial physiology$^{26,27}$. Although we used this inhibitor to specifically elucidate the role of the PMF in $\text{P}_i$ accumulation by cultured *Synechococcus* cells, we compared the CCCP effect with the effect of three other metabolic inhibitors (Supplementary Table 7) on $\text{P}_i$ acquisition by oceanic bacterioplankton to safeguard from potential artefacts. A similar time course of $\text{P}_i$ clearance by the control and 3-(3,4-dichloro-phenyl)-1,1-dimethylurea (DCMU)-treated bacterioplankton demonstrates that DCMU (which reduces photosynthetic electron flow and proton extrusion) slows down the microbial $\text{P}_i$ clearance rate by ~30% (Supplementary Fig. 5). This reduction is consistent with the percentage (~30%) of cyanobacterial cells within the total bacterioplankton. The other three inhibitors completely terminated $\text{P}_i$ clearance by bacterioplankton, as no time course was observed (Supplementary Fig. 5). The effect of N,N’-dicyclohexyl-carbodiimide (DCCD, an ATPase inhibitor) was delayed three times longer compared with 2,5-dibromo-3-methyl-6-
isopropylbenzo-quinone (DBMIB) and CCCP, which halted Pi accumulation after 4 min (Fig. 8a, grey bars).

Likewise, CCCP was a highly effective inhibitor of Pi accumulation by cultured *Synechococcus* cells (Fig. 8a). The time delay of 3–5 min common to the three strains was similar to the 4 min delay of CCCP-treated bacterioplankton. This abrupt halt of Pi accumulation by CCCP-treated *Synechococcus* cells indicates that, as in experiments with bacterioplankton (Fig. 8a), membrane depolarization rather than ATP depletion is behind the inhibition, whereas the generic 4 min delay presumably reflects the time required for CCCP molecules to integrate into cellular membranes. As there was <1 min to incorporate *Pi* into the PFA-fixed macromolecules and even less time to incorporate it into TCA-fixed nucleic acids (Fig. 1d, e), the bulk of the accumulated Pi in CCCP-treated cells (32% and 11% of the total Pi was in the PFA-fixed macromolecules of oceanic bacteria and isolates, respectively) remained labile (Fig. 8a). This comparison of live and fixed cells worked equally well for bacterioplankton and for cultures (Fig. 8a), confirming that in all bacteria tested Pi is first accumulated in the periplasm before being imported into the cytoplasm and assimilated.

The PMF consists of the transmembrane chemical H⁺ gradient (ΔpH) and the transmembrane electric gradient (ΔΨ) that can be sustained by other cations. The protonophore CCCP dissipates both the ΔpH and ΔΨ. To specifically link one of the components to Pi accumulation in the periplasm, we abolished ΔΨ in WH8102 using the three cation-specific ionophores: a K⁺-specific ionophore (Valinomycin), a monovalent cation-specific (e.g., Na⁺ and K⁺) ionophore (Monensin) and a divalent metal-specific (e.g., Mn²⁺, Ca²⁺ and Mg²⁺) ionophore (A23187, Supplementary Table 7), which facilitate the electroneutral transport of cations through a membrane down the chemical gradient. Compared with the control, the three ionophores reduced Pi accumulation into live cells by 0.4×, 1.5× and 2.6×, respectively (Fig. 8b). A significant fraction (20–37%) of the accumulated Pi remained in the periplasm. As relative to the protonophore CCCP, the three ionophores had a minor effect on Pi accumulation by *Synechococcus* cells (Fig. 8b), we conclude that ΔpH rather than ΔΨ is crucial for the periplasmic Pi accumulation. To validate conclusions about energy-dependent accumulation of Pi, drawn from the inhibitor-based experiments, we tested whether Pi accumulation by SAR11, *Prochlorococcus* and *Synechococcus* cells could be energy-stimulated. Conveniently, these bacteria use light energy in their metabolism.

**Light stimulation of Pi accumulation.** To test whether light can energize bacterial Pi accumulation, we compared Pi clearance by oceanic bacterioplankton cells incubated in the light vs. dark
treatments: Pi accumulation into live cells washed with seawater (grey). The logarithmic Y-axis to assist comparing values for the following duplicates indicated as crosses. Horizontal grey lines indicate major ticks of representative experiments. Bars present mean values of technical replicates. a Relative Pi accumulation in bacterioplankton treated with DCCD, DBMIB and CCCP, and Synechococcus strains WH8102, WH7803 and WH8109 treated with CCCP. The time delay is derived from the rate of Pi accumulation, measured for uninhibited control cells. b Relative Pi accumulation in Synechococcus sp. WH8102 cells treated with the K+–specific ionophore Valinomycin (K+), the monovalent metal–specific ionophore Monensin (M+), the divalent metal–specific ionophore A23187 (M++) and the protonophore CCCP (H+). Plots show the results of representative experiments. Bars present mean values of technical duplicates indicated as crosses. Horizontal grey lines indicate major ticks of the logarithmic Y-axis to assist comparing values for the following treatments: Pi accumulation into live cells washed with seawater (grey bars), into PFA-fixed cellular macromolecules (red bars) and into TCA-fixed cellular nucleic acids (blue bars) (a) or into the cytoplasm (white bars) of cells washed with deionized water (b). Source data are provided as a Source Data file.

Fig. 8 Inhibition of bacterial Pi accumulation. a Comparison of the time delay in the inhibition of Pi accumulation between bacterioplankton treated with DCCD, DBMIB and CCCP, and Synechococcus strains WH8102, WH7803 and WH8109 treated with CCCP. The time delay is derived from the rate of Pi accumulation, measured for uninhibited control cells. b Relative Pi accumulation in Synechococcus sp. WH8102 cells treated with the K+–specific ionophore Valinomycin (K+), the monovalent metal–specific ionophore Monensin (M+), the divalent metal–specific ionophore A23187 (M++) and the protonophore CCCP (H+). Plots show the results of representative experiments. Bars present mean values of technical duplicates indicated as crosses. Horizontal grey lines indicate major ticks of the logarithmic Y-axis to assist comparing values for the following treatments: Pi accumulation into live cells washed with seawater (grey bars), into PFA-fixed cellular macromolecules (red bars) and into TCA-fixed cellular nucleic acids (blue bars) (a) or into the cytoplasm (white bars) of cells washed with deionized water (b). Source data are provided as a Source Data file.

Discussion

Accumulation of Pi in the periplasm starts with Pi diffusion from seawater. The theoretical maximal rate of Pi diffusion into the periplasm of a Gram-negative cell depends on a Pi diffusion coefficient and the permeable surface area. The Pi diffusion coefficient is a seawater trait affecting all cells equally. Cells could, however, control their Pi-permeable surface area by varying the number of OM porins. Prochlorococcus and Synechococcus strains respond to Pi limitation by the increased expression of Pi-specific porin genes and the increased abundance of porin proteins. However, the number of these porins in the OM cannot increase indefinitely. Indeed, the maximal Pi clearance rates of oceanic cyanobacterial and SAR11 cells converge towards ~30× below the theoretical maximum. The apparent upper limit to the number of Pi-specific OM porins is probably a result of the competition for space between porins selective for different small solutes, acquisition of which is essential for a living cell.

In contrast to other solutes, Pi somehow accumulates in the periplasm. Synechococcus cells rapidly accumulate labile Pi, which is then steadily internalized and assimilated into macromolecules. The semi-labile nature of the accumulated Pi (hypotonic removal, Figs. 1c, 3 and 5) points to its periplasmic location. Diffusion-driven Pi acquisition can deplete environmental Pi concentrations at a constant clearance rate down to ~10−12 mol l−1 (Fig. 2a). This is feasible only if the periplasmic Pi concentration of Synechococcus cells is <10−12 mol l−1. Even if we assume that the affinity of the Synechococcus PstS subunit for Pi is sufficient to bind Pi at <10−12 mol l−1 concentrations (five orders of magnitude below the determined affinity), then every Pi molecule that enters and stays in the periplasm needs to be instantly bound by a PstS subunit (Fig. 1a). Sequestration of 6.7 × 106 molecules (Fig. 2b) of labile Pi in the periplasm would require an equal number of PstS subunits. The volume of a PstS subunit of 3.5 × 4 × 7 nm dimensions is ~5.1 × 10−23 l; whereas the periplasmic volume of Synechococcus is...
The periplasm is a compartment that is defined by the plasma membrane and contains proteins and enzymes that are not found in the cytoplasm. It is a critical location for the uptake of nutrients, such as phosphate, and for the regulation of cellular functions. In many bacteria, the periplasmic space is a source for Pi acquisition but use their internal energy sources. The high percentage of Prochlorococcus and Synechococcus isolates (e.g., NP_875999, WP_010315318, WP_025922676, WP_002805629 and WP_062436653) indicates that Pi accumulation is linked to cellular energetics and thus destroys ATP-sustained membrane potential, strongly indicates that Pi accumulation is linked to cellular energetics through the PMF rather than being directly driven by ATP through PstSCAB-type transporters (Fig. 1a†). The mutually exclusive conditions (listed above) of PMF-dependent Pi accumulation in the periplasm cannot be explained by PstS-mediated Pi import (Fig. 1a†). Therefore, an alternative mechanism is needed.

An alternative mechanism of PMF-dependent Pi accumulation in the periplasm is a conjecture, because we know little about the organization and functioning of the periplasm in a living bacterial cell. A cell maintains the PMF by extruding protons (H^+) across the plasma membrane against the electrochemical gradient using the energy of respiration and photosynthesis. Accumulation of H^+ in the periplasm would make it acidic relative to both the cytoplasm (pH ~ 7.2) and seawater (pH 8.0–8.3)44,45 (Fig. 1a†). However, it is unlikely that many free H^+ ions would accumulate in the periplasm of marine bacteria, because their OM is permeable to the smallest H^+ ions. Hence, H^+ ions should diffuse out into the environment to be neutralized by >100× excess of OH^− ions in alkaline (pH 8.0–8.3) seawater44, therefore dissipating the membrane potential. To prevent the dissipation of the H^+–based gradient, H^+/Na^+ antiporters may exchange at least some of the periplasmic H^+ ions for Na^+ ions. This substitution could preserve the electrical gradient and support ATP production through H^+/ATP synthases, which can transport Na^+56. Genes for both the H^+/Na^+ antiporter and the Na^+–driven ATP synthase are present in genomes of Prochlorococcus and Synechococcus isolates (e.g., NP_875999, WP_010315318, WP_025922676, WP_002805629 and WP_062436653).

The electric potential between the acidic positively charged periplasm and the alkaline seawater would facilitate diffusion, or more precisely mass transfer, of anions with higher negative charge more strongly than anions with lower negative charge. Thereby, mass transfer through anion-selective pores of HPO_4^{2−} and PO_4^{3−} anions (which comprise 29% and 0.01% of the total Pi in seawater at pH 8.0, respectively57,58) would be particularly favourable. Stronger cationic association of the main seawater anions (e.g., Cl^−, HSO_4^−, HCO_3^− and NO_3^−) would also favour mass transfer of free Pi anions. Once in the H^+–enriched periplasm, the HPO_4^{2−} and PO_4^{3−} anions would associate with one or two H^+ and metal cations to reach pH-dependent speciation equilibrium57,58. Kinetic stability of neutral Pi molecules would explain why disruption of the membrane potential only has a minor immediate effect on the Pi already accumulated in the periplasm (Supplementary Fig. 4).

Neutralization of cations with Pi anions would reduce the PMF, but that could be restored via continuous extrusion of H^+ and cations across the plasma membrane to maintain mass transfer of Pi anions through porins as long as free Pi anions can mass transfer from the environment (Fig. 1a†). Mass transfer would stop when equilibrium is reached, i.e., all Pi anions remaining in seawater are too strongly associated with environmental cations and molecules of water. The strength of their association depends on the total ionic composition of environmental solutions58; in seawater, the association of Pi anions is apparently weak, because we observed bacterial accumulation of Pi anions to continue down to environmental concentrations <10^{-12} mol l^{-1} (Fig. 2) and even <10^{-15} mol l^{-1} (Supplementary Table 4).
Although the periplasm becomes saturated with Pi in 1 h (e.g., 4 × 10^9 P molecules in 2.1 × 10^{-17} l periplasm equals 0.3 mol l^{-1}, Fig. 3b, d), a cell somehow averts precipitation of the Pi salts in the periplasm. To prevent formation of insoluble Pi salts, a cell would need to minimize concentrations of divalent cations (e.g., Ca^{2+} and Mg^{2+}, which associate with Pi to form salts with low solubility) in the periplasm, while balancing the concentrations of monovalent cations (e.g., Na^{+} and K^{+}) and Pi anions to form neutral soluble ion pairs in a way analogous to the common phosphate buffer. Although cation-associated, the H_{2}PO_{4}^{−} and HPO_{4}^{2−} ions remain accessible to PstS-mediated (the PstS subunits have specific affinity to these two anion forms45) import, because they remain soluble (Fig. 1a). Formation of neutral ion pairs would maintain mass transfer of HPO_{4}^{2−} and PO_{4}^{3−} into the periplasm, and prevent H^{+} from escaping into seawater. To simplify the explanations we give below, we suggest to term this periplasmic H^{+}-driven phosphate-cation association—phosphatation.

Maintaining maximal rates of Pi acquisition irrespective of the availability of light energy (Fig. 9) suggests that periplasmic phosphatation is functionally important for marine bacteria as different as cyanobacteria and SAR11 alphaproteobacteria. We propose the following three physiological functions of periplasmic phosphatation: (i) The periplasmic association of Pi, with chemiosmotic cations accumulates not only Pi, but also cations. Accumulation of the latter could be viewed as energy conservation, because H^{+}/Na^{+} import through the plasma membrane can generate ATP59,60. (ii) The high concentration of Pi accumulated in the periplasm could also ensure that the PstS transport system is always saturated with Pi, and operates near its maximal rate. (iii) Periplasmic phosphatation could have an osmotic function. The concentration of Pi in the periplasm can increase by 0.3 mol l^{-1} in just 1 h (e.g., 4 × 10^9 P molecules in 2.1 × 10^{-17} l periplasm, Fig. 3b, d) and reach >0.5 mol l^{-1} within 3 h. Accumulation of an additional 0.5 mol l^{-1} of Pi salt would increase periplasmic osmolarity by ~0.5 osmol l^{-1}. Considering that there are other osmotically active molecules in the periplasm, and that the Pi salt concentration could raise further the periplasmic osmolarity, this would easily exceed the osmolarity of seawater, i.e., ~1 osmol l^{-1} 157. To equilibrate, the resultant osmotic differential water molecules would enter the periplasm increasing its volume and the OM turgor (Fig. 1a), explaining how the OM could work as a load-bearing element61. Therefore, we predict that periplasmic phosphatation is essential for bacterial osmotic regulation—a hypothesis worth experimenting experimentally.

There is little doubt that the cationic attraction of P anions predates life. It was proposed that back in the earliest Archean, positively charged clay particles in oceanic cold alkaline seeps could be viewed as primordial membranes of protocells62. Their positive charge would similarly attract P anions from seawater and this initial enrichment of clay particles with Pi might have been inherited by the first living forms, who gradually engaged Pi in cellular energetics and later genetics. Thus, periplasmic phosphatation is inherently essential for bacterial cells, because it could conserve energy as well as store and attract the vital Pi.

**Methods**

**Environmental sampling.** Data were collected on five oceanographic cruises (Supplementary Table 2) on board the Royal Research Ships James Clark Ross (JCR), James Cook (JC), Discovery III (D) and Discovery IV (DY), and the Research Vessel Maria S. Merian (MSM) in the North Atlantic during Atlantic Meridional Transect cruises AMT17-D299, AMT20-JC039, AMT22-JC079 and AMT27-DY084 in September–October 2005, 2010, 2012 and 2017, respectively, and during the MSM03 cruise in September–October 2006. At predawn, midday and occasional evening station seawater samples were collected from 20 m (a representative depth from the surface mixed layer unaffected by the ship’s movement and contamination) using a 20 × 201 Niskin bottle rosette (Miami, FL, USA) mounted on the stainless steel frame of a conductivity-temperature-depth profiler. Samples were fixed with 200-µl loads of 5 × 10^{-4} M glutaraldehyde. Experiments were performed within 1–2 h after sample collection. The concentrations of bioavailable Pi were determined using isotope dilution, concentration series bioassays22, with carrier-free 32P-labelled or 3P-labelled orthophosphoric acid (Hartmann Analytic GmbH, Braunschweig, Germany). Bioassay experiments were performed in polypropylene, polystyrene and micro-centrifuge tubes (Starlab, Milton Keynes, UK). Flow sorting experiments and experiments with inhibitors were performed in Pyrex glass bottles (Fisher Scientific, Loughborough, UK). To minimize artificial contamination (e.g., P, other nutrients, heavy metals), all plastic-ware, glassware and silicone tubing were soaked in 10% HCl and repeatedly rinsed with DW and sampled seawater.

**Synechococcus strains and growth conditions.** We chose Synechococcus sp. WH8102, because its Pi acquisition and utilization strategies are known1,41. This strain belongs to clade III that is generally considered to be adapted to low-nutrient conditions64, and abundant in Pi-depleted Atlantic waters65. Strain Synechococcus sp. WH8109 is a member of clade II, a clade that dominates tropical and sub-tropical regions of the world’s ocean66, tolerates intermediate nutrient-depleted conditions66,68, and lives in nutrient-richer upwelling waters69. It was reasonable to expect that these strains accumulate Pi extracellularly, because they grow better under Pi-depleted rather than under Pi-replete conditions41,90. To elucidate whether the Pi accumulation was specific to cyanobacterial ecotypes that had evolved to cope with Pi deprivation, we also included an opportunistic strain Synechococcus sp. WH7803 (clade V) that reaches a maximal growth rate when supplemented with ≥ 10^{-6} mol l^{-1} Pi90.

**Experimental procedures of Synechococcus sp. WH8102 and Synechococcus sp. WH7803 were axenic. An axenic culture of Synechococcus sp. WH8109 strain was established by flow sorting. Live bacterial cells in a mixed culture were stained with 0.1 µg ml^{-1} Hoescht 33342 (final concentration) and Synechococcus sp. WH8109 cells were flow cytometrically differentiated from other bacteria by their specific autofluorescence. Using a custom-built MoFlo XDP instrument91, WH8109 cells were flow-sorted in batches of 100-200 cells directly into sterile tubes with ASW medium. These tubes were placed into an illuminated growth chamber and incubated until growth became evident by colour.

**Cell enumeration and flow sorting.** Cultured Synechococcus cells were counted live, unstained, to assess their cell density during laboratory experiments (e.g., see Fig. 5c). Oceanic samples were fixed with PFA (1% w/v, final concentration), stained with SYBR Green I DNA dye69 at 20 °C in the dark for 1 h and flow cytometrically counted and sorted22 (Supplementary Fig. 1). Bacterioplankton cells flow-sorted during the D299 and JC039 cruises were taxonomically identified using DAPI fluorescence in situ hybridization92. High nucleic acid-containing bacteria with virtually undetectable chlorophyll autofluorescence21 were identified as Prochlorococcus. Low nucleic acid-containing bacteria were identified as SAR11 alphaproteobacteria. Oceanic Synechococcus were identified flow cytometrically based on their specific orange autofluorescence93.

**Western blot analyses.** Cells from an exponentially growing Synechococcus WH8102 culture (OD_{670nm} ≥ 0.25) were starved for Pi to induce PstS synthesis. After 60 h incubation, 800 ml cells were pelleted by centrifugation, washed twice with 20 ml ASW, and subjected to a mild hypotonic shock in 60 ml ASW containing 0.5 g l^{-1} yeast extract and were contaminant free. To maintain trace Pi conditions, glassware used for Pi-free cultivation and experiments was soaked in 10% HCl and thoroughly rinsed with DW.

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Pulse-chase experiments. Exponentially growing Synechococcus sp. WH8102 cells (0.3–3 × 10^6 cells ml^{-1}) were collected on a 0.2 μm pore size Nuclepore track-etched filter membrane (Whatman International Ltd, Maidstone, UK) mounted in a filter holder (Swinney, Millipore, Ireland) and washed with 40 ml of P-free ASW (ASW–P) at a flow rate of 2 ml min^{-1} using a syringe pump (KD Scientific, Holliston, MA, USA). The washed cells were pulsed with 10 μl H_3^{32}PO_4, followed by 10 μl ASW–P at a rate of 0.5 ml min^{-1} (Supplementary Table 6). For treatments in spin columns, 0.3 ml of cells were incubated in a column, spun down at 5000 x g for 1 min and washed thrice with 0.1 ml of ASW–P. The radioactivity was determined separately for the cells retained in the spin column insert and for the effluent in the collection tube. To fix cells, 0.5–1.5 ml cells were fixed with 1% PFA, incubated at ambient temperature for 1 h and filtered on 0.2 μm pore size polycarbonate filters. Filters were rinsed twice with 4 ml DW, placed in 20 ml scintillation vials (Meridian, Epsom, UK), mixed with 10 ml scintillation cocktail (Goldstar, Meridian) and radioassayed using a low-activity liquid scintillation analyser (Tri-Carb 1380 TR/SL, PerkinElmer, Beaconsfield, UK) with QuantaSmart® software. To assess their reproducibility, the pulse-chase experiments were repeated thrice. The results of two experiments are presented in Fig. 3.

Extracellular P, labelling of Synechococcus cells. Cells washed of free P, were re-suspended in 20 ml ASW–P, supplemented with 80–160 Bq ml^{-1} of H_3^{33}PO_4 or H_3^{32}PO_4 and incubated in a glass bottle under standard cultivation conditions for 1–3 h. The labelled cells were collected on 0.2 μm pore size Nuclepore filters and effluent was sampled to determine the efficiency of P clearance (Supplementary Table 6). Extracellularly pre-labelled Synechococcus cells were washed free of residual P, with 20 ml ASW–P, and re-suspended in solutions appropriate for further experiments.

Retention of the accumulated P. Pre-labelled Synechococcus cells were treated with enzymes (Supplementary Table 6) or re-suspended in a range of solutions (Supplementary Tables 5 and 8) using either a 0.2 μm pore size PVDF membrane spin column or a 0.2 μm pore size Nuclepore membrane filter fitted in a 13 mm diameter plastic filter holder. For treatments in spin columns, 0.3–0.5 ml treated cells were incubated in a column, spun down at 5000 x g for 1 min and washed thrice with 0.1 ml of an appropriate washing solution. The radioactivity was determined separately for the cells retained in the spin column insert and for the effluent in the collection tube. Alternatively, cells were collected on a Nuclepore membrane, washed with 10 ml of a treatment solution at a rate of 0.5–1 ml min^{-1} and the radioactivity determined for the filter membrane only. The activity of the protease Proteinase K in ASW was confirmed using the PDQ protease assay (Athena Environmental Sciences, Inc., Baltimore, MD, USA). To assess their reproducibility, all experiments were repeated independently at least thrice.

Inhibition experiments. Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and added to samples to a final concentration of 0.05–1 × 10^{-4} mol l^{-1} (Supplementary Table 7) similarly with the 32P pulse. To provide a control, samples were supplemented with the appropriate amount of DMSO. Accumulation of ^32P in DMSO-amended samples and control samples without DMSO addition was similar (Shapiro–Wilk normality test P = 0.932, paired t-test t = −0.155 with 8 degrees of freedom, two-tailed P-value 0.881). Samples were incubated in the light under ambient conditions and sub-samples were withdrawn periodically to determine the total microbial P accumulation. To assess their reproducibility, all experiments were repeated independently at least thrice.

Microbeam synchrontron X-ray fluorescence analysis. These measurements were performed at the Diamond Light Source, the UK National Synchrotron Facility. Beamline I18 (Microfocus Spectroscopy) was used to measure the amount of P in cells of Prochlorococcus cyanobacteria (range 1–5 × 10^6 cells) and Synechococcus sp. strain WH8102 (0.1–1 × 10^6 cells) (Supplementary Table 3). Cells were flow-sorted onto a 0.2 μm pore size polycarbonate filters (P free) using the custom-built MoD XRF instrument^68. The filters with dried sorted cells were mounted on a custom-made sample holder and the cell-containing filter areas (~1000 × 2000 μm) were probed with a beam spot of 30 × 30 μm² in a helium atmosphere (Supplementary Fig. 3a-c). An approximation of the number of photons on the sample was determined by aligning a flat reference silicon nitride wafer with known mass deposition of a number of metals. This calculated photon flux applied to the cells and accounting for differences in sample matrix, density and volume, leads to an estimated mass per pixel for the cells. From these values, the total number of P atoms was derived for each cell preparation by integrating signal from all P containing pixels using PyMCA X-ray Fluorescence Toolkit (http://pymca.sourceforge.net) and ImageJ 1.50i software (http://imagej.nih.gov/ij/). Using the cultured Synechococcus cells as calibrants, sensitivity (±2 × 10^4 cells) and linear range (±5 × 10^5) of the method were determined.

The determined P content was 4.14 ± 1.06 × 10^8 P atoms per cell for oceanic Prochlorococcus equivalent to 1.3–3.1 g (accessions: ASM1148v1, ASM1246v1, ASM1264v1, ASM1564v1, ASM1566v1, ASM1858v1, ASM1829v1, ASM17598v1, ASM792v1 and ASM1146v1). The extracellularly accumulated P of cells was removed during sorting by the sheath fluid of low osmotic concentration (~0.03 osmol l^{-1}), which strips the extracellularly bound P (Figs. 1, 3 and 5). Therefore, only intracellular phosphorus was measured for the sorted cells. In comparison, the P content calculated for an exponentially growing extracellular P-free Synechococcus sp. WH8102 cell was 1.9 ± 0.16 × 10^10 P atoms, which is ~4 times the genomic P content (ASM19597v1).

Microscopy. Bacterioplankton cells were imaged and their dimensions measured^68 (Supplementary Fig. 3d-f and Supplementary Table 1). Cells of Synechococcus sp. WH8102 were imaged using a LSM780 Confocal Laser Scanning Microscope (Carl Zeiss, Jena, Germany), using 488 nm (Ar) and 594 nm (HeNe) laser lines.

Statistics and reproducibility. Each experiment was repeated independently at least three times and often more than ten times.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The auxiliary data collected on research cruises are archived indefinitely at the British Oceanographic Data Centre (BODC) and are available at www.bodc.ac.uk. The p-SXR data are available from Open Science Framework at http://osf.io/6RH2V. All other data supporting the findings of this study are available within the paper and its Supplementary Information files. The source data underlying Figs. 2–4 and 6–9, and Supplementary Figs. 2, 4 and 5 are provided as a Source Data file.

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Author contributions
M.V.Z. and N.A.K. conceived the project, designed and performed the experiments, analysed and interpreted the data and co-wrote the manuscript. K.G. designed and performed µ-SXRF phosphorus measurements and analysed data. D.J.S. contributed expert advice. M.V.Z. and D.J.S. provided funding. All authors critically edited the manuscript.

Competing interests
The authors declare no competing interests.

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