RESEARCH ARTICLE

Comparison of phosphate uptake rates by the smallest plastidic and aplastidic protists in the North Atlantic subtropical gyre

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Received 28 January 2011; revised 9 June 2011; accepted 15 June 2011.
Final version published online 14 July 2011.

DOI:10.1111/j.1574-6941.2011.01160.x

Editor: Riks Laanbroek

Keywords
phosphorus limitation; protozoa; picophytoplankton; radiotracer; picoeukaryotes; bacterioplankton.

Abstract

The smallest phototrophic protists ($< 3\,\mu m$) are important primary producers in oligotrophic subtropical gyres – the Earth's largest ecosystems. In order to elucidate how these protists meet their inorganic nutrient requirements, we compared the phosphate uptake rates of plastidic and aplastidic protists in the phosphate-depleted subtropical and tropical North Atlantic (4–29°N) using a combination of radiotracers and flow cytometric sorting on two Atlantic Meridional Transect cruises. Plastidic protists were divided into two groups according to their size (< 2 and 2–3 μm). Both groups of plastidic protists showed higher phosphate uptake rates per cell than the aplastidic protists. Although the phosphate uptake rates of protist cells were on average seven times ($P < 0.001$) higher than those of bacterioplankton, the biomass-specific phosphate uptake rates of protists were one fourth to one twentieth of an average bacterioplankton cell. The unsustainably low biomass-specific phosphate uptake by both plastidic and aplastidic protists suggests the existence of a common alternative means of phosphorus acquisition – predation on phosphorus-rich bacterioplankton cells.

Introduction

As a necessary component of DNA, RNA and lipids and with a key role in bioenergetics, phosphorus is a vital biogenic element. In the ocean, it is one of the key elements, including nitrogen and iron, controlling primary production. It has even been argued that phosphorus is the ultimate limiting nutrient for primary production in the ocean (Tyrrell, 1999). Although the North Atlantic subtropical gyre is depleted in phosphorus (Wu *et al.*, 2000; Zubkov *et al.*, 2007; Mather *et al.*, 2008), phytoplankton photosynthesis and growth are mainly limited by the availability of inorganic nitrogen, although there is evidence for a degree of colimitation by phosphorus and iron (Mills *et al.*, 2004, 2008; Davey *et al.*, 2008).

The nomenclature of the smallest protists discussed here is quite variable in the literature (Zubkov & Tarran, 2008; Casey *et al.*, 2009; Jardillier *et al.*, 2010). For example, plastidic protists are often named picoeukaryotes or picophytoplankton, while aplastidic protists are called protozoa. To simplify reading, we use the terms plastidic and aplastidic protists to distinguish between protists that are considered to contain chloroplast(s) (plastidic protists, Plast) or lack chloroplast(s) (aplastidic protists, Aplast), respectively. The former are further divided into two groups of smaller and larger cells according to their size: $< 2\,\mu m$ (Plast-S) and 2–3 μm (Plast-L). The term bacterioplankton (Bpl) is used throughout the text to include all bacterial (pigmented and nonpigmented) and archaeal cells that were present in the described sample.

In the North Atlantic subtropical gyre *Prochlorococcus* and low nucleic acid containing bacterioplankton (LNA), dominated by SAR11 *Alphaproteobacteria*, control ~95% of the bioavailable phosphate (Zubkov *et al.*, 2007). With regard to protists, previous studies have focused exclusively on plastidic protists. In the Sargasso Sea, plastidic protists were reported to take up at most 3% of the total available phosphate (Casey *et al.*, 2009). Owing to their low

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abundance in the central North Atlantic, our provisional data suggested that the plastidic protests $< 3\,\mu m$ contribute ~1% to total microbial phosphate uptake (Zubkov *et al.*, 2007). This low contribution, in the context of strong bacterioplankton competition for resources, raises the question of whether these organisms can satisfy their phosphorus requirements by taking up only dissolved phosphate.

Here, we systematically determined and compared the phosphate uptake rates of plastidic, aplastidic protists and bacterioplankton using $H_3^{33}PO_4$ tracer in the central North Atlantic subtropical and tropical gyre in order to assess the dependence of the smallest plastidic protists on phosphate taken directly from seawater.

Materials and methods

Sampling

Seawater samples were collected before sunrise (predawn) from a depth of 20 m with 20-L Niskin bottles mounted on a sampling rosette equipped with a conductivity–temperature–depth profiler. Bottle content was decanted into a 20-L polycarbonate carboy, washed with 10% hydrochloric acid and rinsed with sampled seawater. Radiotracer experiments commenced no later than 30 min after sample collection. A total of 19 experiments were conducted in the subtropical and tropical North Atlantic during two Atlantic Meridional Transect (AMT) cruises, 18 and 19, on board the Royal Research Ships (R.R.S.) James Clark Ross and James Cook in October–November 2008 and October–November 2009, respectively (Fig. 1).

Cell enumeration

Bacterioplankton and protist cell abundances were determined by flow cytometry (FACSort, Becton Dickinson, UK). Samples were fixed with a final concentration of 1% w/v paraformaldehyde and stained with SYBR Green I DNA dye (Marie *et al.*, 1997; Zubkov *et al.*, 2007). Multifluorescent beads of 0.5 and 1.0 μm (fluoresbrite microparticles, Polysciences) were used as internal standards for fluorescence and flow rates (Zubkov & Burkill, 2006).

Determination of ambient phosphate concentration, microbial uptake rates and turnover time using bioassays

^{33}P-orthophosphoric acid ($> 111\,TBq\,mmol^{-1}$, Hartmann Analytic GmbH, Germany) was added at a standard concentration of 0.1 nM and diluted in parallel experiments with nonlabelled (cold) Na_2HPO_4 using the dilution series of 0.4, 0.8, 1.6, 2.4, 3.2 and 4.0 nM. Triplicate samples (1.6 mL) were incubated in 2-mL screw top-capped sterile polypropylene microcentrifuge tubes in the dark and at *in situ* temperatures. A sample was fixed at each of 20, 40, 60 and 80 min, by adding a 20% paraformaldehyde solution

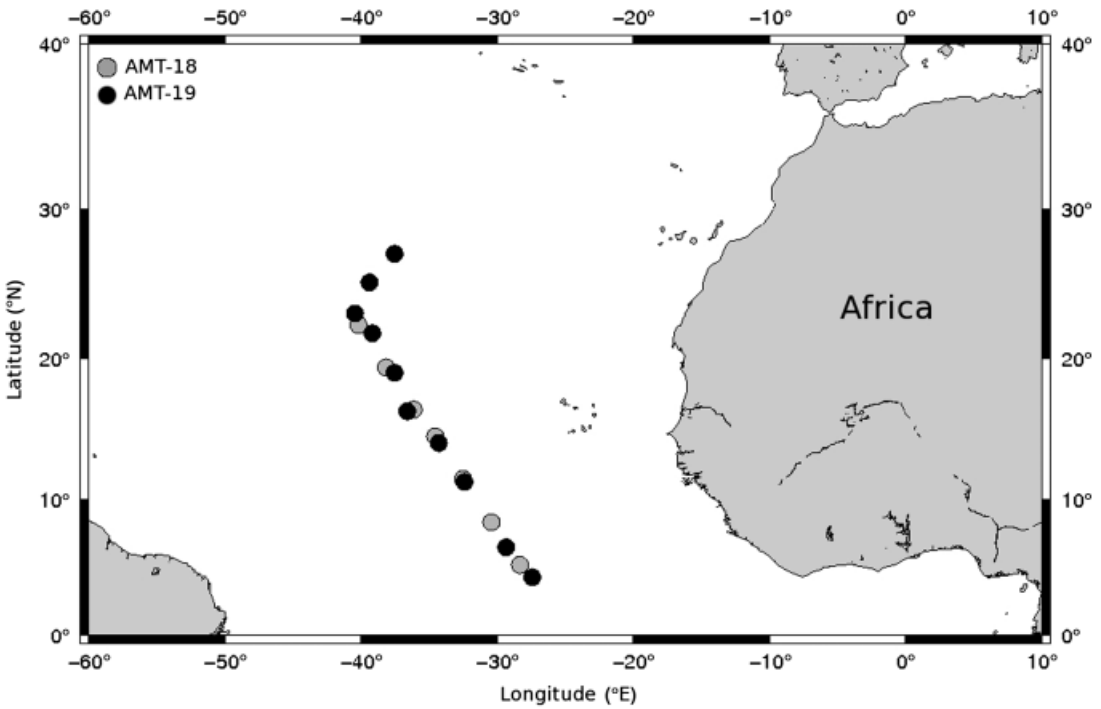

Fig. 1. Cruise track of AMT-18 and AMT-19 undertaken in October/November 2008 and 2009, respectively.

(1% w/v final concentration). The sampled particulate material was harvested onto 0.2-μm polycarbonate filters (Poretics Corporation) and washed with two 5-mL aliquots of a solution of 0.5 M LiCl and 1 mM phosphate ($LiCl–PO_4$ buffer), adjusted to pH 9 (Grillo & Gibson, 1979). Before harvesting, the filters were autoclaved in the same buffer. Radioactivity retained on the filters was determined in Becquerel (Bq) using a liquid scintillation counter (Tri-Carb 3100, Perkin Elmer). For a detailed explanation of the calculations to estimate the ambient phosphate concentration, microbial uptake rates and turnover times, please refer to Zubkov *et al*. (2007).

Uptake of radiolabelled phosphoric acid by different microbial groups using flow cytometric sorting

In order to determine the cell-specific microbial uptake rates of phosphate, 250-mL seawater samples were inoculated with 0.08 nM of $H_3^{33}PO_4$, incubated in the dark at *in situ* temperatures for 2–4 h in glass bottles (Schott, Fisher Scientific, UK). To estimate the total microbial phosphate uptake, 250, 500 and 750 μL of the fixed samples were filtered on 0.2-μm polycarbonate filters. Several subsamples of 1.6 mL were taken between 0.25 and 4 h, fixed with paraformaldehyde and filtered through 0.2-μm polycarbonate filters to determine whether the total microbial uptake of phosphate is linear with time. At 11 stations on AMT-19, the uptake of phosphate by flow-sorted plastidic protists, aplastidic protists and bacterioplankton cells was compared in 120-mL samples incubated for 2 and 4 h in order to assess the linear dependence of cellular phosphate uptake upon time.

Paraformaldehyde-fixed radiolabelled samples were stored at 4 °C before being processed within 10 h. Flow cytometric sorting was carried out as described previously (Zubkov & Tarran, 2008). Four different types of cells were flow sorted: Bpl, Plast-S, Plast-L and Aplast. Sorted cells were collected onto 0.2-μm polycarbonate filters, washed with deionized water to remove residual tracer and placed into scintillation vials to which 5 mL of scintillation cocktail (GoldStar, Meridian, UK) was added. On AMT-19, 0.8-μm polycarbonate filters were used to collect the protist cells in order to reduce a number of possible by-sorted bacterioplankton cells. Sorting controls showed that 0.8-μm filters retained very few labelled bacterioplankton cells, with radioactivity measurements statistically similar to the background. In all cases, the scintillation vials were radioassayed as described above.

In order to estimate the relative contribution of each population to the total phosphate uptake, the cell-specific phosphate uptake rates were multiplied by the number of cells per millilitre of the respective populations and the percentage contribution to the total phosphate uptake was subsequently calculated. The phosphate uptake of the different populations analysed was summed up and compared with the total microbial phosphate uptake measured (Zubkov *et al*., 2007). Linear regression analysis showed a 1 : 1 relationship between those two values ($R^2 = 0.97$, $P < 0.001$), confirming that all ^{33}P tracer taken up by microorganisms was accounted for. Leakage of the intercellular phosphorus out of cells after paraformaldehyde fixation could result in a decrease of ^{33}P tracer amount in cells (Larsen *et al*., 2008). We could not correct for that loss here. Consequently, the presented values should be regarded as lower estimates.

Biomass normalization of phosphate uptake rates was carried out because of the considerable differences in cell biomass between bacterioplankton and protist cells. All biomass estimates were based on literature values of cell volume or cell size, which were converted into biomass using the conversion factor of 0.2 pg C μm^{-3} (Waterbury *et al*., 1986). If cell size was used to calculate cell volume, a spherical cell shape was assumed. The mean average diameter of bacterioplankton cells was assessed on earlier AMT cruises using a size fractionation technique (Zubkov *et al*., 2000) as 0.46 ± 0.14 μm. Using the abovementioned conversion factors, their biomass was estimated as 0.011 ± 0.0035 pg C per cell. The biomass of Plast-S and Plast-L was calculated using the values published by Jardillier *et al*. (2010); the cell size was on average 1.8 ± 0.3 and 2.8 ± 0.9 μm, respectively, yielding a biomass of 0.6 ± 0.1 and 2.3 ± 0.7 pg C per cell, respectively. According to the flow cytometric analyses, aplastidic protists had the same DNA content and light scatter as large plastidic protists. Thus, we assumed that Aplast and Plast-L had approximately the same cell size and hence biomass.

To estimate C : P values for protists, we determined the absolute phosphate uptake using the bioassay data in combination with flow cytometric cell sorting data (Zubkov *et al*., 2007). C : P values are based on earlier measurements of CO_2 fixation (Grob, C., unpublished) and were combined with the phosphate uptake rates measured in this study (for additional information, see Zubkov *et al*., 2007).

Data analyses

All comparisons between different microbial groups were carried out at one station and not between different stations. This approach avoided the use of absolute values, because the conversion factors, for example ambient concentration of phosphate or the total microbial community uptake rates derived from the bioassay experiment, would be applied to all the compared groups. A *t*-test or a paired *t*-test was used to compare datasets using SIGMAPLOT 11.0 software (Systat Software Inc.). The computational results were tested for statistical significance at $P < 0.05$.

Results

The phosphate concentrations measured on AMT-18 were lower than those measured on AMT-19 (*t*-test, $P = 0.003$). On average, the phosphate concentrations were 1.3 ± 0.7 and 6.2 ± 4.5 nM, respectively. No significant difference (*t*-test) were observed between the average total microbial uptake rates of phosphate, 0.07 ± 0.06 and 0.26 ± 0.35 nM h^{-1}, on AMT-18 and AMT-19, respectively. The phosphate turnover times of 44 ± 61 h on AMT-18 and 75 ± 73 h on AMT-19 were not significantly different. The SDs reflect latitudinal variability rather than methodological error. The detailed bioassay data will be presented in a separate communication. Also, no significant differences in the average microbial cell numbers were observed between the two cruises. $870\,000 \pm 290\,000$ and $900\,000 \pm 270\,000$ Bpl cells, 380 ± 230 and 690 ± 390 Plast-S cells, 1300 ± 400 and 930 ± 230 Plast-L cells and 930 ± 320 and 710 ± 340 Aplast cells were present per millilitre of seawater at 20 m on the AMT-18 and the AMT-19 cruise, respectively.

The total microbial uptake of phosphate increased linearly with time, confirming the adequacy of the incubation conditions (Fig. 2). We also compared the phosphate uptake rates of microorganisms in samples incubated for 2 and 4 h. A significant increase in phosphate uptake by flow-sorted bacterioplankton as well as that by protist cells was proportional to time (Fig. 3), demonstrating consistency in the group-specific rates determined.

Owing to their larger size, phosphate uptake of protist cells was significantly higher than phosphate uptake of bacterioplankton cells (Fig. 4a–c). On average, Plast-S and Aplast cells took up 6.4 times more ^{33}P-phosphate per hour per cell than bacterioplankton cells. Plast-L cells took up 8.6 times more phosphate than bacterioplankton cells. However, when biomass differences between the cells were taken into account, the results were dramatically different: the biomass-specific uptake rates of protists were only 5–15% of the biomass-specific rates of bacterioplankton (Fig. 4d–f).

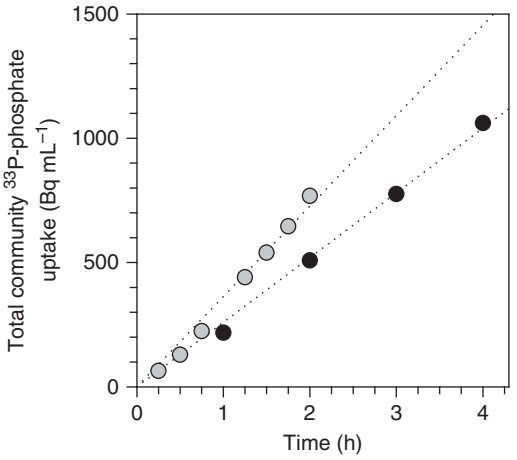

Fig. 2. Total microbial ^{33}P-phosphate uptake over time using two representative stations as examples. The colours indicate the different cruises on which the data were collected: AMT-18 (grey) and AMT-19 (black).

A comparison of the plastidic protist groups with the aplastidic group (Fig. 4) revealed a significant difference between Plast-L and Aplast cells (paired *t*-test, $P < 0.001$). Plast-L cells showed, on average, 58% higher per cell phosphate uptake rates than Aplast cells. On the other hand, no significant difference between Plast-S and Aplast (paired *t*-test) could be found in cell-specific phosphate uptake rates. However, taking the biomass into account, Plast-S took up on average 150% and 230% more phosphate than Plast-L and Aplast, respectively (paired *t*-test, $P = 0.009$ and $P = 0.005$, respectively).

No latitudinal trend could be observed in the cellular phosphate uptake rates of either bacterioplankton or protists (Fig. 5). Phosphate uptake in the central North Atlantic was entirely dominated by bacterioplankton (Fig. 6). On average, the summed phosphate uptake rates for the three described protist groups contributed only $1.9 \pm 0.9\%$ to the total microbial phosphate uptake. Owing to their higher abundance, the Plast-L group took up significantly more phosphate than the Aplast group ($1.2 \pm 0.75\%$ and $0.5 \pm 0.3\%$, respectively, paired *t*-test, $P = 0.001$). The fraction of phosphate acquired by Plast-S is statistically similar to that of the Aplast group ($0.5 \pm 0.3\%$) (Fig. 6). Hence, there is a similar difference in the phosphate uptake contribution between the Aplast and the Plast-L populations.

Discussion

Oligotrophic oceanic gyres are globally important ecosystems covering over 30% of the Earth's surface. Plastidic protists ($< 3\,\mu m$) contribute significantly to primary production in these North Atlantic subtropical waters (Li, 1994; Jardillier *et al.*, 2010). However, the phosphate uptake rates of the same plastidic protists in the North Atlantic gyre are very low (Zubkov *et al.*, 2007 and this study). The minor contribution of plastidic protists to the total microbial phosphate uptake is not restricted to the central North Atlantic, but was also shown in the western North Atlantic (Casey *et al.*, 2009). The fact that ambient phosphate concentrations and cellular phosphate uptake rates do not show latitudinal trends (Fig. 5) suggests that the mechanisms of phosphorus acquisition by microbial populations are similar in this large area of the North Atlantic. Within the eukaryotic populations, phosphate uptake is dominated by the plastidic protists. Biomass-corrected uptake rates show similar contributions of Plast-S and Plast-L populations while Aplast contribute significantly less (paired *t*-test, $P = 0.001$). This higher uptake rates are a result of the higher abundance of plastidic protists in comparison with

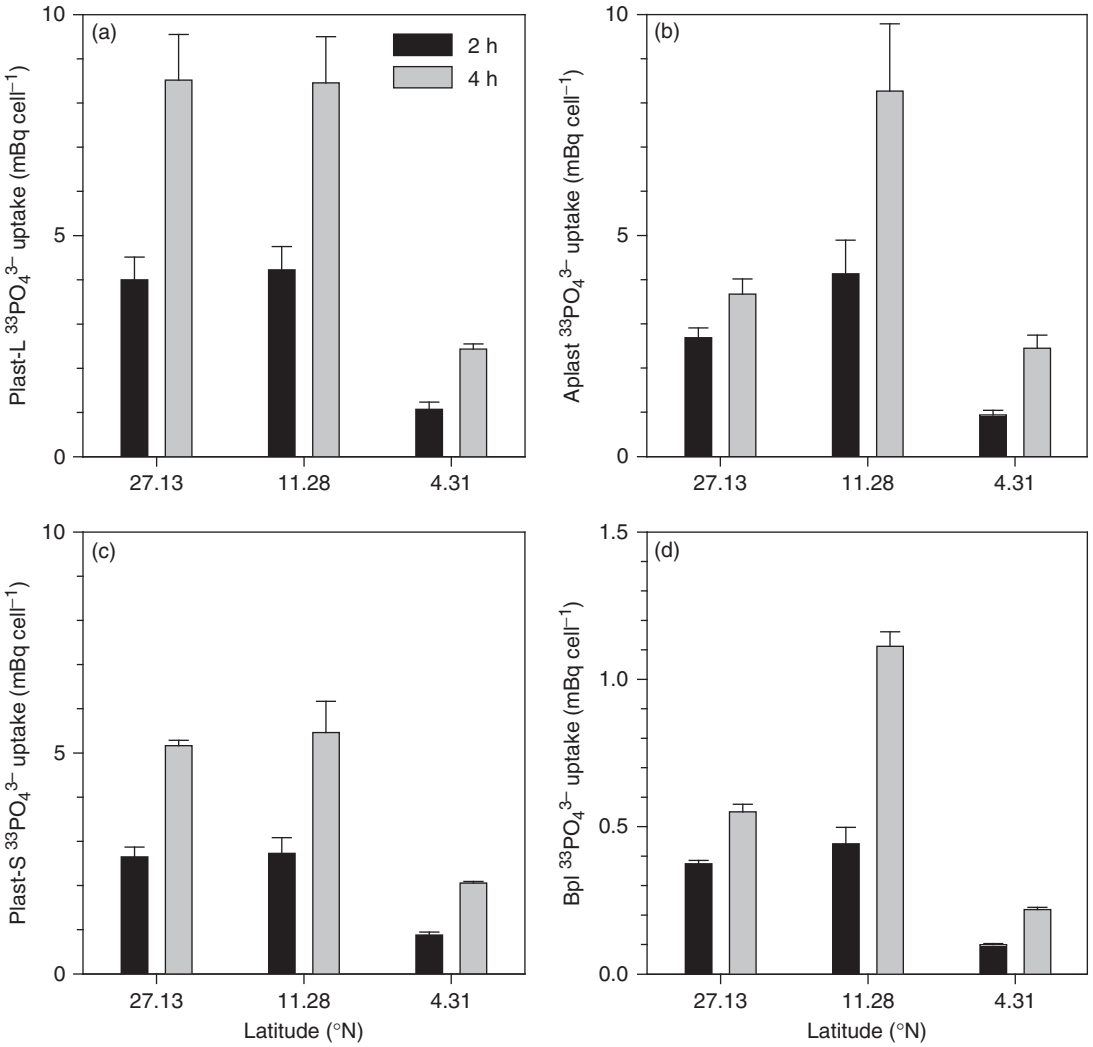

Fig. 3. Per cell ^{33}P-phosphate uptake after 2 h (black) and 4 h (grey) measured at three different stations. (a) Large plastidic protists (Plast-L), (b) aplastidic protists (Aplast), (c) small plastidic protists (Plast-S) and (d) average bacterioplankton cells (Bpl). Please note the different scale for the bacterioplankton plot (d).

aplastidic protists. Although phosphate uptake rates by protist cells are considerably higher than by the mean bacterioplankton cells, biomass normalization reveals protists' apparent inability to match the efficiency of bacterioplankton acquisition of phosphate (Fig. 4).

The C : P values of individual groups of plastidic protists can vary significantly, for example in nutrient-replete cultures, the ratio is ~200 : 1 for *Prasinophyceae* and ~50 : 1 for *Prymnesiophyceae* (Ho *et al.*, 2003; Quigg *et al.*, 2003). Phosphate uptake rates by plastidic protists, measured on AMT-17 (Zubkov *et al.*, 2007) and combined with historical measurements of CO_2 fixation rates (Li, 1994), showed C : P values of approximately 1000 : 1, suggesting the potential phosphorus limitation of plastidic protist cells. Based on the present results and parallel CO_2 fixation measurements (Grob, C., unpublished), the C : P values should be even higher, at approximately 2000–4000 : 1. The small contribution of protists to the total microbial phosphate uptake rates (Fig. 6), combined with their lower biomass-normalized phosphate uptake rates compared with bacterioplankton (Fig. 4), suggest that protists should be outcompeted by bacterioplankton when it comes to the uptake of dissolved phosphate. Hence, protists need to satisfy their phosphorus demands using other sources.

Protists can acquire phosphorus in different ways. For example, symbionts could help to procure nutrients such as phosphate (Cole, 1982). However, given the small size of the protists studied here, such symbionts should be bacteria that in order to survive should take up phosphate in ways similar to their free-living counterparts. For this reason, phosphate uptake by symbionts should be directly measurable using the methods applied here as a high biomass-specific rate of

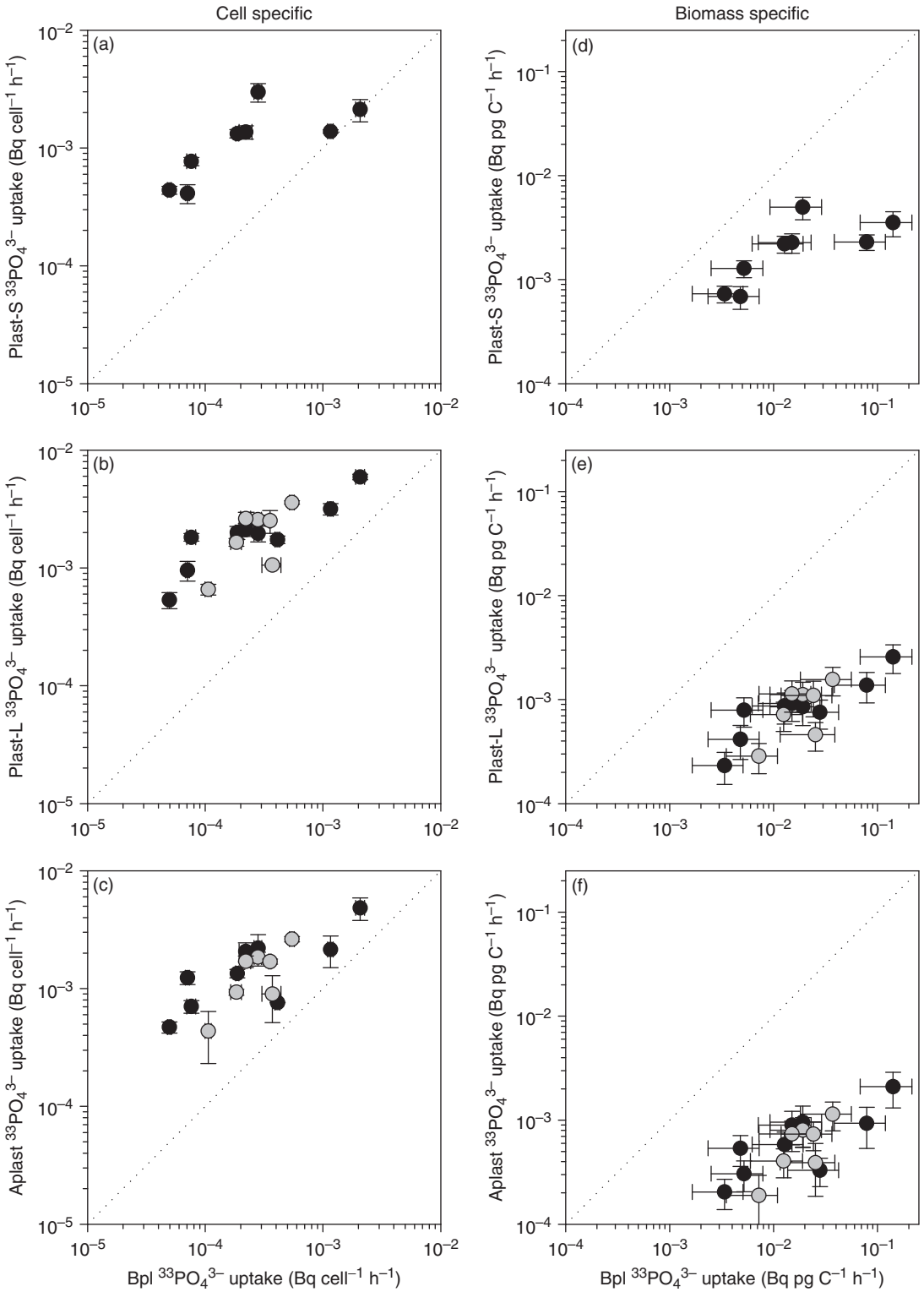

Fig. 4. Cell-specific (left column) and biomass-specific (right column) ^{33}P-phosphate uptake rates for individual protist groups in comparison with bacterioplankton. (a, d) Small plastidic protists (Plast-S), (b, e) large plastidic protists (Plast-L), (c, f) aplastidic protists (Aplast). The colours indicate the different cruises on which the data were collected: AMT-18 (grey) and AMT-19 (black).

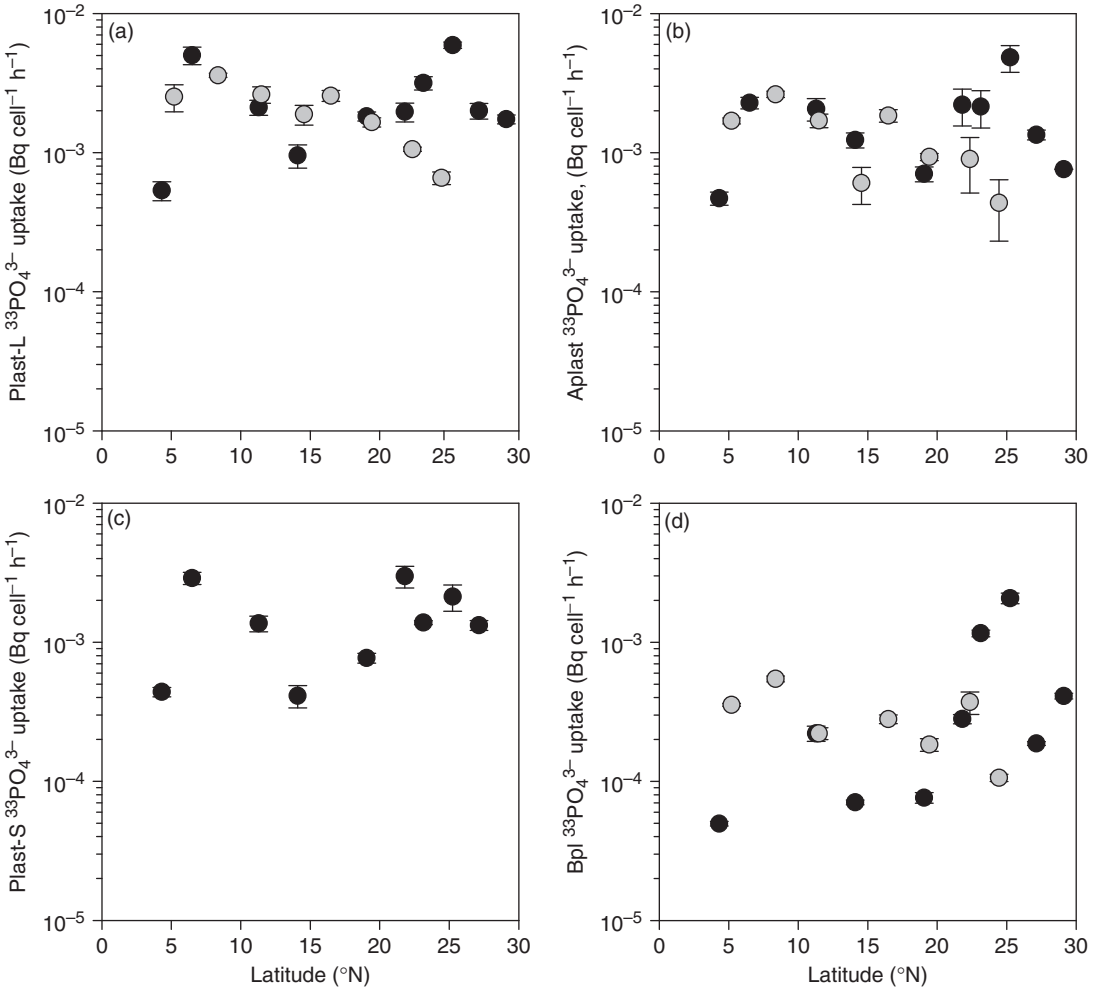

Fig. 5. Latitudinal variability in cellular ^{33}P-phosphate uptake rates of protist groups and bacterioplankton. (a) Large plastidic protists (Plast-L), (b) aplastidic protists (Aplast), (c) small plastidic protists (Plast-S) and (d) bacterioplankton (Bpl). The different colours indicate on which cruise the data were collected: AMT-18 (grey), and AMT-19 (black).

their protist hosts, which is not the case. Other sources of phosphorus that might be available to protists, but not included in the current study, are various organic phosphorus compounds including nucleotides and phosphonates. In the upper water column, dissolved organic phosphorus can represent a considerable fraction of the total available phosphorus (Wu *et al.*, 2000). Using ATP as a proxy for organic phosphate, we have shown previously that uptake of organic phosphate plays only a minor role in the total phosphate uptake, due both to low uptake rates as well as slow turnover (Zubkov *et al.*, 2007 and our unpublished data).

Biogenic phosphonates comprise up to a quarter of the measurable, high-molecular-weight dissolved organic phosphorus in marine waters and could serve as an alternative phosphorus source (Clark *et al.*, 1998). For example, it was shown that phosphonates comprise up to 10% of the cellular phosphorus pool of *Trichodesmium erythraeum* in culture (Dyhrman *et al.*, 2009). The C–P bond is, however, very recalcitrant and, up to now, an ability to degrade phosphonates has only been found in prokaryotes (Adams *et al.*, 2008) rather than in protists. Therefore, it is unlikely that protists import large quantities of dissolved phosphonates. An alternative strategy of reducing cellular phosphorus demands, mastered by marine bacteria, is the synthesis of molecules analogous to phospholipids, for example sulpholipids (Van Mooy *et al.*, 2006). Hypothetically, protists could do the same, and it would be worth analysing their lipid composition. Unfortunately, the physiology of the smallest protists is still largely unknown mainly because representative cultures have not been established.

Both plastidic and aplastidic protists can also obtain phosphorus by preying on bacterioplankton. A combination of photosynthesis and feeding on bacterioplankton or other

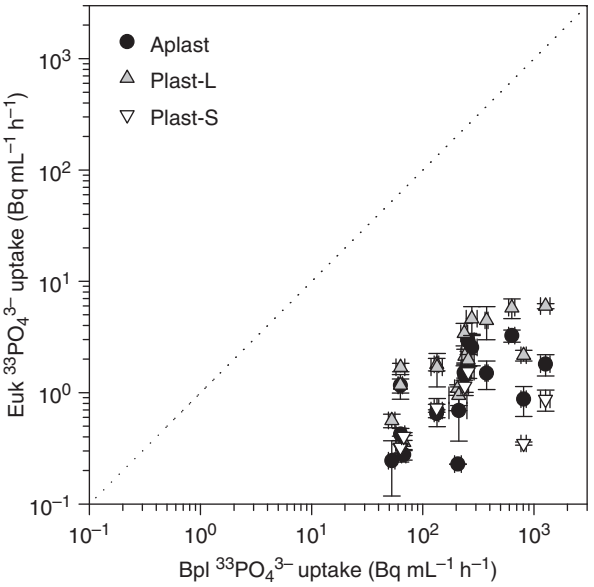

Fig. 6. Population-specific ^{33}P-phosphate uptake rates of aplastidic protists (Aplast, black), large plastidic protists (Plast-L, grey), and small plastidic protists (Plast-S, white), in comparison to bacterioplankton (Bpl).

protists, i.e. mixotrophy, is widespread among protists (e.g. Stoecker *et al.*, 1997; Unrein *et al.*, 2007). For example, in the temperate North Atlantic, plastidic and aplastidic protists can consume similar quantities of bacterioplankton (Zubkov & Tarran, 2008). It is most likely that aplastidic protists acquire all their phosphorus, including the ^{33}P tracer (Fig. 4c), from prey. On the other hand, plastidic protists could still satisfy a part of their phosphorus requirements by taking up dissolved phosphate. By assuming that aplastidic and plastidic protists have comparable physiological requirements for phosphorus, we can estimate the proportion of phosphate taken up by plastidic protists from seawater as the difference between the amount of ^{33}P tracer taken up by them and the amount of ^{33}P tracer measured in aplastidic protists. This calculation indicates that only one third of the phosphorus needed by Plast-L cells could originate from phosphate dissolved in seawater. Because the Plast-S cells are smaller than the Plast-L and Aplast cells, they have a proportionally larger cell surface area compared with cell volume. Because phosphate transporters are located on the cell surface, Plast-S cells can have more phosphate transporters in relation to their cell volume and hence can acquire phosphate more rapidly per unit biomass. Hence, correcting for biomass differences between the Aplast and Plast-S cells, Plast-S could recover at most two third of their phosphorus demands by taking up dissolved phosphate. Furthermore, bacterivory helps to explain the unrealistically high C : P values computed for plastidic protists because a major fraction ($< 99.9\%$) of the intracellular phosphorus pool would remain unlabelled during a 2–4-h incubation before a bacterioplankton cell fell prey to a protist.

In summary, in the North Atlantic subtropical and tropical gyre, the smallest protists seem to be outcompeted by bacterioplankton in the uptake of phosphate from seawater. Direct uptake of phosphate seems insufficient to satisfy the physiological demands of the two size groups of the smallest plastidic protists. An alternative means of phosphorus acquisition should explain the abundance of protists in the open ocean. Feeding on bacterioplankton appears to be the most likely means of phosphorus acquisition by plastidic protists. Direct quantification of their bacterivory will be an unequivocal proof of this hypothesis.

Acknowledgements

We gratefully acknowledge the captains, officers and crew aboard the R.R.S. James Clark Ross and the R.R.S. James Cook for their help during the two cruises. Furthermore, we would like to thank Dr Glen Tarran for his critical comments on the manuscript. This study was supported by the UK Natural Environment Research Council (NERC) through a research grant (NE/E016138/1) and by the National Oceanography Centre Core Programme.

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