Small pigmented eukaryotes play a major role in carbon cycling in the P-depleted western subtropical North Atlantic, which may be supported by mixotrophy

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Abstract
We found that in the phosphate (PO4)-depleted western subtropical North Atlantic Ocean, small-sized pigmented eukaryotes (P-Euk; < 5 μm) play a central role in the carbon (C) cycling. Although P-Euk were only ~5% of the microbial phytoplankton cell abundance, they represented at least two thirds of the microbial phytoplankton C biomass and fixed more CO2 than picocyanobacteria, accounting for roughly half of the volumetric CO2 fixation by the microbial phytoplankton, or a third of the total primary production. Cell-specific PO4 assimilation rates of P-Euk and nonpigmented eukaryotes (NP-Euk; < 5 μm) were generally higher than of picocyanobacteria. However, when normalized to biovolumes, picocyanobacteria assimilated roughly four times more PO4 than small eukaryotes, indicating different strategies to cope with PO4 limitation. Our results underline an imbalance in the CO2:PO4 uptake rate ratios, which may be explained by phagotrophic predation providing mixotrophic protists with their largest source of PO4. 18S rDNA amplicon sequence analyses suggested that P-Euk was dominated by members of green algae and dinoflagellates, the latter group commonly mixotrophic, whereas marine alveolates were the dominant NP-Euk. Bacterivory by P-Euk (0.9 ± 0.3 bacteria P-Euk−1 h−1) was comparable to values previously measured in the central North Atlantic, indicating that small mixotrophic eukaryotes likely exhibit similar predatory pressure on bacteria. Interestingly, bacterivory rates were reduced when PO4 was added during experimental incubations, indicating that feeding rate by P-Euk is regulated by PO4 availability. This may be in response to the higher cost associated with assimilating PO4 by phagocytosis compared to osmotrophy.

Marine single-cell eukaryotic microorganisms, or protists, are ubiquitous and diverse and are major contributors in oceanic food webs and biogeochemical cycles (Sherr et al. 2007). Determining their taxonomic identity, the extent to which they contribute to carbon (C) and nutrient cycling, and how they respond to environmental change remain among the major challenges in oceanography (Ohtsuka et al. 2015; Keeling and Campo 2017). Answering these questions is fundamental to understand marine ecosystem functioning and to predict how the marine environment will respond to global climate changes (Worden et al. 2015). Although the abundance, distribution, and ecological roles of marine protists have been the focus of many studies (Ohtsuka et al. 2015), there is still much to be learned, particularly for the smaller sized eukaryotes (i.e., picoeukaryotes [2–3 μm] and nanoeukaryotes [2–20 μm], hereafter designated as small eukaryotes) that are increasingly being recognized as important to oceanic C cycling as primary producers and consumers (Massana et al. 2009; Cuvelier et al. 2010; Grob et al. 2011; Massana 2011; Hartmann et al. 2012; Worden et al. 2015; Rii et al. 2016a,b). Despite recent progress in the examination of marine protists using modern molecular tools, major gaps remain in our understanding of these understudied eukaryotic microorganisms, particularly in comparison to marine prokaryotes (Caron et al. 2017; Keeling and Campo 2017). Although the reasons for this slow progress are manifold, as discussed in detail elsewhere (e.g., Caron et al. 2017; Keeling and Campo 2017), the application of omics tools to small eukaryotes represents a major challenge with limited success (Keeling 2013). Understanding the metabolism and functions of small-sized protists (answering the question “who is doing what?”) and examining how environmental factors affect
their role in biogeochemical transformations will therefore require combining different approaches.

Protists can be autotrophic (photosynthetic), heterotrophic (feeding on other organisms), or mixotrophic (having a combined mode of nutrition, autotrophy and heterotrophy). Accurately describing the rates of activity of protists (e.g., primary production, grazing, and nutrient uptake) is extremely important to C and nutrient cycle models (Thingstad and Havskum 1999; Ward and Follows 2016). In most oligotrophic and mesotrophic areas of the ocean, primary production is dominated by picophytoplankton cells (< 2–3 μm) composed of the picocyanobacteria genera Prochlorococcus (Chisholm et al. 1988) and Synechococcus (Waterbury et al. 1979) and a poorly defined assemblage of autotrophic small eukaryotes, i.e., picoeukaryotes and nanoeukaryotes (Worden et al. 2004). Although greatly outnumbered by picocyanobacteria, due to their larger cell size, small autotrophic eukaryotes can contribute significantly to C biomass (Grob et al. 2007; Buitenhuis et al. 2012; Bock et al. 2018), primary production (Li 1994; Jardillier et al. 2010; Grob et al. 2011; Rii et al. 2016a,b), and nutrient uptake (Fawcett et al. 2011; Popendorf and Duhamel 2015). Given the enormous areal extent covered by the open ocean, any changes in primary production by small eukaryotes are likely to have a global impact. Yet, little is known about the role of small autotrophic eukaryotes in C and nutrient dynamics in the oligotrophic ocean, where nutrients are scarce. In addition, pico- and nano-size heterotrophic eukaryotes play an important role in controlling C cycling and microbial food webs. Through feeding, they control bacterioplankton abundances and can alter the microbial community composition via selective grazing; as prey for marine zooplankton, they channel organic C and nutrients to higher trophic levels; and while they can regenerate nutrients supporting phytoplankton growth, they can also contribute to osmotrophic assimilation of nutrients and compete with other microbial plankton for limited resources (Pernthaler 2005; Sherr et al. 2007; Massana et al. 2009). However, heterotrophic protists’ contribution to nutrient uptake has been seldom studied in comparison to other groups of microbial plankton, especially in the open ocean. Recent advances in flow cytometry cell sorting of samples incubated with stable or radioactive molecules have been key to improve our understanding of biogeochemical rates and processes at the microbial group level (Lomas et al. 2011). Yet, those data are scarce, especially in the most oligotrophic areas of the ocean, and the relative contribution of different plankton groups to C and nutrient assimilation have only been studied independently, preventing a full understanding of the processes and roles of key organisms in elemental cycling and stoichiometry.

Despite their importance in C cycling, the functional diversity of small eukaryotes, mostly uncultured, remains poorly known, and recent attempts to fill this gap underscore the urgent need to disassemble the black box of bacterial grazers (Frias-Lopez et al. 2009; Massana et al. 2009; Not et al. 2009; Massana 2011). In addition to the more frequently studied groups of strictly phototrophic or phagotrophic small plankton species, the importance of mixotrophic bacterivory in comparison to obligate heterotrophic bacterivory for small eukaryotes was only recently demonstrated (Zubkov and Tarran 2008; Hartmann et al. 2011, 2012, 2013). However, the mechanisms driving mixotrophy by microbial eukaryotes are not widely acknowledged or understood (Stoecker et al. 2017). A mixed mode of nutrition may be particularly advantageous in oligotrophic ecosystems because nutrients are often limiting to phototrophic microbial eukaryotes that acquire nutrients by osmotrophic pathways (Unrein et al. 2007; Hartmann et al. 2012). Through the consumption of prey, small mixotrophic eukaryotes may obtain nutrient supplements to grow. Recent models indeed predict that in nutrient-deficient marine environments, the nanoplanckton mixotroph community acquires nutrients by grazing (Ward and Follows 2016; Edwards 2019). Considering the high cost of phagotrophy compared to osmotrophy, we hypothesize that ingestion rates by small mixotrophic eukaryotes should be regulated by ambient nutrient concentration (Christaki et al. 1999).

This work aims to fill major gaps in our understanding of the diversity and function of small eukaryotes in the oligotrophic ocean, with emphasis on their ecological role in C and nutrient dynamics in comparison to the picocyanobacteria Prochlorococcus and Synechococcus, the most abundant phototrophic cells in the ocean. Here, we investigated the contribution of picocyanobacteria and small eukaryotes (mostly < 5 μm) to primary production (CO2 fixation rates) and phosphate (PO4) uptake along a North–South transect in the western subtropical North Atlantic Ocean. To achieve this goal, we used seawater incubations with radiolabeled molecules followed by fluorescence-activated cell sorting (FACS) of major microbial plankton groups. We also measured in situ PO4 uptake capabilities (i.e., the ability of microorganisms to assimilate PO4 as a function of concentration, a.k.a. Michaelis–Menten parameters) for each group, to determine adaptations to maximize nutrient assimilation in the PO4-depleted western subtropical North Atlantic Ocean. To improve our understanding of the composition of microbial eukaryotes, we conducted complementary microscopy analyses of morpho-groups and phylogenetic analyses of 18S rDNA sequences that were obtained for different functional groups separated by FACS. We also investigated mixotrophy by small eukaryotes by examining feeding rates of bacterioplankton by small pigmented eukaryotes (P-Euk) separated by FACS. Finally, we tested the role of PO4 availability in regulating phagotrophy by those small mixotrophic eukaryotes.

Material and methods

Sample collection

Environmental samples were collected during the Bermuda Atlantic Time-Series Study (BATS) validation cruise BValS0 in the western subtropical North Atlantic Ocean in September 2015, along a transect from Bermuda to Puerto Rico extending from 33°N to 22°N (Fig. 1; Table 1). Water was collected from 20-m depth using acid-washed, ultrapure water and sample-rinsed bottles and kept in subdued fighting until processing
(within 15 min). We chose to focus on a depth within the mixed layer based on previous work showing that mixotrophic nanoplankton in the Sargasso Sea were more abundant in surface waters than deeper in the euphotic layer and that it may be an adaptation to low nutrient availability (Arenovski et al. 1995). Chlorophyll \(a\) (Chl \(a\)) concentration was determined following the BATS procedure (http://batsftp.bios.edu/BATS/bottle/), which uses fluorometric analysis of samples extracted in 100% acetone (Herbland et al. 1985).

Radioisotope incubation experiments

\(\text{PO}_4\) assimilation and \(\text{CO}_2\) fixation rates

Seawater was collected into acid washed, ultrapure water and sample rinsed, clear polycarbonate incubation bottles. \(\text{PO}_4\) assimilation rates were measured in triplicate 70-mL samples with \(\sim\) 259 kBq of added \(^{33}\text{P}-\text{PO}_4\) (Perkin Elmer #NEZ08000; carrier free), incubated for 30 min to 1 h. \(\text{CO}_2\) fixation rates were measured in duplicate 70-mL samples with \(\sim\) 17 MBq of added \(^{14}\text{C}-\text{sodium bicarbonate}\) (Perkin Elmer #NEC086H000, 1.6 GBq mmol\(^{-1}\)), incubated from dawn to dusk. Samples were incubated under simulated light and temperature conditions measured at the sampling site. A killed control sample was also prepared by adding paraformaldehyde (PFA; 2% final concentration prepared with electron microscopy grade 16% aqueous solution, Electron Microscopy Sciences) at least 15 min before introducing the radioisotope, in order to account for unincorporated radioactivity. At the end of incubation, samples were fixed with PFA (2% final, for 15 min in the dark), and triplicate 20-\(\mu\)L aliquots were sampled to measure the total radioactivity added (with \(\beta\)-phenylethylamine for \(^{14}\text{C}\) samples). The total

![Fig. 1. Station locations (3–14) plotted on 8-d average MODIS chlorophyll for 14 to 21 September 2015. White areas have no data because of cloud cover.](image-url)
microbial activity was determined by filtering a 3-mL aliquot through a 0.2-μm, pore-size polycarbonate membrane filter (Nuclepore). To reduce unincorporated $^{33}$P-PO$_4$, the membrane filter was placed onto a filter type HA soaked in 100 mmol L$^{-1}$ KH$_2$PO$_4$, then rinsed three times with ~1 mL of 0.2-μm filtered seawater. To remove unincorporated $^{14}$C-sodium bicarbonate, the filter was acidified with 0.5 mL of 1 mol L$^{-1}$ HCl for 24 h. To determine plankton groups specific uptake rates, a 20-mL aliquot was passed through a 0.2-μm polycarbonate membrane filter under gentle vacuum filtration, and the remaining volume from the 70-mL incubation bottle was passed through a 0.8-μm polycarbonate membrane filter. The 0.2- and 0.8-μm filters were stored in separate scintillation vials with 4 mL of scintillation cocktail (Ultima Gold LLT, Perkin Elmer) added. To remove unincorporated radioactivity and total microbial activity were assayed by liquid scintillation counting using a Packard Tri-Carb 3110 TR liquid scintillation counter with ultra-low-level option kit, in 7-mL plastic scintillation vials (Simport) with 4 mL of scintillation cocktail (Ultima Gold LLT, Perkin Elmer) added.

Turnover times ($T_{PO_4}$, h) were calculated by dividing the total radioactivity added (Bq L$^{-1}$) by the rate of radiolabel uptake into the particulate fraction (Bq L$^{-1}$ h$^{-1}$). PO$_4$ assimilation rates (nmol PL$^{-1}$ h$^{-1}$) were calculated by dividing PO$_4$ concentration by $T_{PO_4}$. Samples were collected for soluble reactive phosphorus (SRP) determination (hereafter PO$_4$) and were processed according to the MAGIC-SRP protocol (Karl and Tien 1992) for low-level PO$_4$ in seawater, but results fell mostly below our detection limit (3 nmol L$^{-1}$). Instead, we used PO$_4$ concentration estimated from concentration series bioassays following the method of Wright and Hobbie (1966). Briefly, seawater samples were amended with nonradioactive PO$_4$ to target additions of 0, 5, 10, 25, 50, 75, and 150 nmol PO$_4$ L$^{-1}$, spiked with $^{33}$P-PO$_4$, incubated and sampled as described above. The resulting $T_{PO_4}$ values were plotted against a corresponding concentration of PO$_4$, and extrapolated using linear regression ($T_{PO_4} = a x PO_4 + b$) to estimate the ambient concentration ($Sn = b/a$), which represents an upper estimate of ambient concentrations as detailed in Zubkov and Tarran (2005). Results from these bioassays were also used to calculate the Michaelis–Menten kinetic parameters for PO$_4$ assimilation rates ($V_{max}$, the maximum rate at saturating substrate concentration and $K_m$, the half-saturation constant).

**Bacterivory rates by mixotrophic small eukaryotes**

Bacterivory rates by small-sized P-Euk were measured following the protocol by Zubkov and Tarran (2008). In order to test if phosphate availability affects feeding rates, bacterivory rates were estimated in untreated seawater or with the addition of a saturating concentration of PO$_4$ (hereafter control and $+$PO$_4$, respectively) at three stations (3, 8, and 14). Bacterioplankton cells were pulse-chase-labeled with $^{35}$S-methionine, which was shown to provide more robust rates of bacterivory by protists than using $^{3}H$-leucine (Hartmann et al. 2012). Seawater was collected into two 500-mL acid-washed, ultrapure water and sample-rinsed, clear polycarbonate incubation bottles and 1 mmol L$^{-1}$ KH$_2$PO$_4$ was added in one of the bottles. Samples were then spiked with 0.4 nmol L$^{-1}$ $^{35}$S-methionine (Perkin Elmer #NEG0097000, 43.48 TBq mmol$^{-1}$) and incubated for 1.5 h (pulse) as described above. Triplicate, 20-μL samples were taken to measure the total radioactivity added. To chase the $^{35}$S-methionine, 0.4 μmol L$^{-1}$ of nonradioactive L-methionine was added and 115 mL subsamples were taken after 2, 4, 6, and 9 h of incubation and fixed with 2% PFA (final concentration). Then a 1.8-mL aliquot was transferred into a cryovial, and the leftover was gently concentrated under gentle vacuum filtration to a final volume of 4.5 mL using a 0.8-μm polycarbonate membrane as described above. This concentrated sample was transferred into a separate cryovial for the measurement of bacteria and P-Euk tracer uptake rates, respectively, using flow cytometry cell sorting (see below). In parallel, a 1.6-mL sample aliquot was sampled and filtered onto a 0.2-μm polycarbonate membrane filter every 15 min for 3 h, then every hour until the end of the 9-h experiment to verify that there was no significant change in the radioactivity in the particulate fraction after the chase. To check for potential osmotic uptake of $^{35}$S-methionine by small-sized protists during the chase phase, additional “chase–pulse” experiments (vs. “pulse–chase” experiments) were set up by initially spiking 250-mL seawater samples with 0.4 μmol L$^{-1}$ unlabeled methionine and subsequently spiking with 0.4 nmol L$^{-1}$ $^{35}$S-methionine after 1.5-h incubation. Subsamples were fixed after 3- and 9-h incubations. The radioactivity in “chase–pulse” sorted cells was similar to the background, meaning that osmotic uptake of tracers during the chase was insignificant (Hartmann et al. 2012).

**Flow cytometry analyses**

Major microbial plankton groups were characterized by flow cytometry using a BD Influx flow cytometer (BD Biosciences). Duplicate 1.8-mL samples were fixed with PFA (electron microscopy grade, 0.25% final concentration) for 15–20 min in the dark at room temperature, flash frozen in liquid nitrogen, and preserved at −80°C until analysis. The sheath fluid consisted of a sodium chloride solution filtered in-line through a 0.22-μm Sterivex™ filter unit. Reference beads (Fluoresbrite, YG, 1-μm) were added to each sample to maintain proper alignment and focus of the instrument. *Prochlorococcus*, *Synechococcus*, and pigmented protists (P-Euk, sometimes referred to as plastidic protists in the literature) were discriminated in unstained samples based on their chlorophyll (red) fluorescence and forward scatter (FSC, size) signatures (Supporting Information Fig. S1). The high phycocerythrin (orange) signal in *Synechococcus* was used to distinguish them from *Prochlorococcus* and P-Euk. Using a FSC detector with small particle option and focusing a 488 plus a 457 nm laser (200 and 300 mW solid state, respectively) into the same pinhole permitted the resolution of dim surface *Prochlorococcus* group from background noise (Duhamel et al. 2014). Additionally, nonpigmented protists (NP-Euk, sometimes referred to as aplastic protists in the literature) were discriminated from P-Euk in SYBR...
Green-stained samples using a chlorophyll (red) vs. SYBR Green (green) fluorescence plot, as described in Bock et al. (2018). The average cell size of *Prochlorococcus*, *Synechococcus*, and P-Euk was estimated based on relationships between particle diameter and FSC (Casey et al. 2009), using SPHERO™ Size Standard Kits. The P-Euk and NP-Euk were essentially < 5 μm (Supporting Information Fig. S1). In SYBR Green-stained samples, the P-Euk population comprised subpopulations A and B, with P-Euk_A characterized by lower red and green fluorescence and lower FSC than P-Euk_B (Jardillier et al. 2010).

For cell sorting, the influx flow cytometer was set at the highest sorting purity (1.0 drop single mode), and potential attached cells were discarded using a pulse width vs. FSC plot. The drop delay was calibrated using Accudrop Beads (BD Biosciences) and verified manually by sorting a specified number of reference beads onto a glass slide and counting the beads under an epifluorescence microscope. Performance was validated by sorting *Prochlorococcus* and *Synechococcus* from natural samples and reanalyzing the sorted cells flow cytometrically to confirm sort purity and recovery. Three to four proportional numbers of cells from the same incubation sample were sorted for each target population (e.g., 25,000, 50,000, 15,000 Synechococcus, and 400, 800, 1600, and 3200 P-Euk and NP-Euk). Sorted cells were assessed by liquid scintillation analysis following previously published protocols (Talarmin et al. 2011; Duhamel et al. 2012; Rii et al. 2016a). The 14C-labeled samples were acidified with 1 mL of 2 mol L⁻¹ HCl for 24 h to remove any unincorporated 14C-sodium bicarbonate. For each group, at least three samples were sorted and regression analysis between the number of cells sorted and the radioactivity taken up by the sorted cells was used to calculate the per cell activity (dpm cell⁻¹). Radioactivity in sorted cells from the PFA-killed control samples (dpm cell⁻¹) was deduced from radioactivity in the sorted cells from the respective samples (dpm cell⁻¹). The cell-specific assimilation rate (nmol cell⁻¹ h⁻¹) was calculated by dividing the radioactivity per cell (dpm cell⁻¹) by the total microbial activity (dpm L⁻¹) measured in the same sample and then multiplied by the total microbial assimilation rate at ambient substrate concentration (nmol L⁻¹ h⁻¹). Bacteriervy by P-Euk (bacteria P-Euk⁻¹ h⁻¹) was calculated as follows: Bacteriovir = (P₁,2 × B₁,2⁻¹ − P₁ × B₁⁻¹) × (T₂ − T₁)⁻¹, with P₁,2 and B₁,2 the average cell-specific radioactivity (based on triplicate measurements) by the P-Euk and bacteria, respectively, after 9 h incubation, and P₁ and B₁ the same after 4 h incubation; T₁ and T₂ are time points of the experiment (4 and 9 h, respectively). Due to the nature of the pulse-chase experimental design, the activity in B₁ and B₂ was in most cases the same and a cumulative mean could be used, as suggested by Hartmann et al. (2012).

Alkaline phosphatase activity

Alkaline phosphatase activity (APA) was measured following a modification of the Dyhrman and Ruttenberg (2006) protocol. Seawater samples were collected onto two separate filters with 0.2- and 0.8-μm porosities, chosen to tease apart activity by the bacterioplankton (i.e., bacteria and cyanobacteria) and the eukaryotes-enriched fractions (0.2–0. 8 μm and > 0.8 μm, respectively). Filters were stored frozen at −20°C until analysis (< 1 month). Samples were processed using the fluorogenic phosphatase substrate 4-methylumbelliferyl phosphate (MUF-P, Sigma-Aldrich) at saturating concentration (10 μmol L⁻¹; Casey et al. 2009). Fluorescence was measured on a Horiba FluoroMax-4 spectrophuorometer at several time points within the linear range of the assay. A standard curve using 4-methylumbelliforone (MUF, Sigma-Aldrich) was used to calculate MUF-P hydrolysis rates (Duhamel et al. 2011). We report volumetric (nmol L⁻¹ h⁻¹) and Chl a normalized rates (nmol h⁻¹ μg Chl a⁻¹) of APA.

Small eukaryotes diversity

Each seawater sample was prefilted through 8-μm polycarbonate filter and concentrated using a CellTrap™ filter cartridge (MEM-TEQ Ventures) and a peristaltic pump. About 4 liter of seawater was concentrated to a 1300 μL final volume, which was mixed with 300 μL of 0.2-μm filtered glyTE (33 μL 100X TE pH 8.0, plus 100 μL of ultrapure water, plus 167 μL molecular-grade glycerol). The mixture contained within a cryovial was flash frozen using liquid nitrogen and kept at −80°C until analysis. P-Euk and NP-Euk were separately sorted into Eppendorf tubes by flow cytometry as described in Flow Cytometry Analyses section. DNA was extracted from each sorted population of cells—ranging between 2000 and 8000 cells—using a PureLink™ Genomic DNA kit (ThermoFisher Scientific). Extracted DNA samples were sent to Mr. DNA Lab (Shallowater, TX) for 18S rDNA V4 and V8-9 amplicon library preparations and sequencing on the Illumina’s MiSeq platform. Note, unlike the study by Kim et al. (2016), amplicon libraries were prepared in a single polymerase chain reaction (PCR) reaction, a standard procedure at Mr. DNA Lab. Otherwise, PCR primers targets V4 and V8-9 regions of eukaryotic 18S rDNA as well as read processing follow the procedures of Kim et al. (2016). Briefly, raw reads were quality trimmed using Trimmomatic ver. 0.36 (Bolger et al. 2014), and paired reads were merged using FLASH (Magoc and Salzberg 2011). The merged reads were demultiplexed based on unique barcode IDs. PCR primer sites were removed using cutadapt (Martin 2011). Read clustering and Operational Taxonomic Unit (OTU) annotation were performed by using the USEARCH pipeline (Edgar 2013) with the Silva SSU Ref database (Quast et al. 2013). OTUs annotated as animals (e.g., human) or plants were discarded for further analyses. After read quality trimming, merging, and removal of animal and plant sequences, total read counts per sample ranged from 25 to 150 K. Analyses of amplicon sequence data hinted possible sample cross contamination at a low level, which may have been due to Illumina’s barcode switching (Wright and Vetsigian 2016) and/or cross-talk during cell sorting. We thus used a set of criteria to minimize the influence of data cross contamination. First, when a given OTU had counts in the paired P-Euk and NP-Euk samples, unless the difference was less than 10-fold, its presence was only assumed for the sample
with the higher read counts. Note that even with such conservative approach, a total combined level of contamination per sample was estimated to be typically below 5%, not exceeding 10% of total reads. Additionally, only those OTUs with relative abundance of 1% or greater were considered for further analyses. These select, relatively abundant OTUs comprised 85–97% of the total reads (Fig. 2). For these select OTUs, their respective centroid reads were analyzed by BLAST (Morgulis et al. 2008; Supporting Information Data S1).

**Microscopy**

One liter samples were gently concentrated onto 0.8-μm polycarbonate filter under low vacuum, stirring gently down to 95 mL, to which 5-mL of 50% glutaraldehyde (aqueous solution of electron microscopy grade, Electron Microscopy Sciences) was added. Samples were kept refrigerated until analysis within 1 month. Ten-milliliter aliquots of the thoroughly mixed, glutaraldehyde-fixed microplankton samples were centrifuged to sediment the cells, and the overlying supernatant was removed leaving 0.5 to 1 mL of fluid. The sedimented pellet was gently resuspended in the remaining 1 mL of supernatant and stained with Lugol’s iodine. Samples of the thoroughly suspended stained sample (20 μL) were taken with a micropipette and deposited as a droplet in the base of an Uttermohl viewing chamber and examined using a Nikon Diaphot inverted compound microscope with phase contrast optics. The content of each 20-μL droplet was scanned field-by-field exhaustively at ×400, and each individual microplankton observed was measured in the micron range, using an ocular reticule, and assigned to a broad taxonomic morpho-group (e.g., coccolithophore, diatom, and dinoflagellate). Based on the volume of the water sample examined in the total sample of droplets for each morpho-group, the counts of each microplankton morpho-group were converted to densities (number mL⁻¹). Examples of individuals within each morpho-group were photographed using a digital camera, and representative images of each morpho-group were assembled in a plate of micrographs illustrating the range of microplankton that were observed (Supporting Information Fig. S2).

**Graphical representations and statistical analyses**

Graphical representations and statistical analyses were performed using Prism 6 for Mac OS X version 6.0h. Michaelis–Menten kinetic parameters were determined using the Michaelis–Menten model in Prism 6. For cell enumeration, cytograms were analyzed using FCS Express 6 Flow Cytometry Software (De Novo Software).

**Results**

**Description of the study area**

Stations sampled (n = 7) along a longitudinal transect from 33.25°N to 22.16°N near 64.14°–65.37°W were characterized by high sea surface temperatures (29.0°C ± 1.0°C), which tended to increase from north to south (Table 1). PO₄ concentrations at 20-m depth were always < 3 nmol L⁻¹ and the turnover time of PO₄ was short (TPO₄ = 5.7 ± 0.1 to 7.5 ± 0.1 h, n = 5), indicating PO₄ limitation (Moutin et al. 2008). To further assess...
Table 2. Cell abundances of microbial plankton groups (*Prochlorococcus*, *Synechococcus*, pigmented microbial eukaryotes, and nonpigmented microbial eukaryotes—Pro, Syn, P-Euk, and NP-Euk, respectively, ×10^3 cells mL^-1) enumerated by flow cytometry and of total microphytoplankton and dinoflagellates (Phyto and Dino, respectively, ×10^3 cells L^-1) enumerated by light microscopy.

| Sta. | Pro | Syn | P-Euk* | P-Euk_A | P-Euk_B | NP-Euk | Phyto | Dino |
|------|-----|-----|--------|---------|---------|--------|-------|------|
| 3    | 52.0| 9.2 | 0.7    | 0.3     | 0.5     | 1.0    | N.D.  | N.D. |
| 5    | 37.4| 7.3 | 0.8    | 0.2     | 0.5     | 0.8    | N.D.  | N.D. |
| 6    | 9.1 | 5.5 | 0.6    | 0.2     | 0.4     | 0.5    | 1.00  | 0.3  |
| 8    | 5.4 | 3.5 | 0.8    | 0.2     | 0.5     | 0.4    | 1.20  | 0.10 |
| 10   | 4.8 | 2.8 | 0.6    | 0.1     | 0.5     | 0.4    | N.D.  | N.D. |
| 11   | 4.4 | 2.5 | 0.6    | 0.1     | 0.5     | 0.4    | 0.30  | 0.04 |
| 14   | 14.3| 3.6 | 0.6    | 0.2     | 0.4     | 0.6    | 3.10  | 0.50 |

N.D., not determined.

*Note that while P-Euk were enumerated from unstained samples, P-Euk_A and P-Euk_B were enumerated from stained samples.

the P-status of the microbial community, we measured APA. Total particulate (>0.2 μm) APA was relatively high, ranging from 1.6 to 2.3 nmol L^-1 h^-1 (1.8 ± 0.3 nmol L^-1 h^-1, n = 5) with 20% to 39% (29% ± 7%, n = 5) measured in the size fraction >0.8 μm (i.e., the eukaryotes-enriched fraction). Chl a concentration at 20 m was 0.046 ± 0.005 μg L^-1 (n = 7; Table 1). Chl a normalized total particulate APA ranged from 31.8 to 49.7 nmol h^-1 μg Chl a^-1 (41.6 ± 7.9, n = 5), with higher values in the southern part of the transect, where the lowest PO₄ concentrations were estimated (Table 1).

Microbial community composition

Microbial abundances were generally dominated by *Prochlorococcus*, followed by *Synechococcus* (18.2 ± 18.9 and 4.9 ± 2.5 × 10^3 cell mL^-1, respectively, n = 7) and tended to decrease from north to south with the exception of Sta. 14 where abundances rose back (Table 2). This is comparable to values measured at 20 m at BATS in September between 2014 and 2016 (32.1 ± 45.5 and 5.1 ± 2.4 × 10^3 cell mL^-1, for *Prochlorococcus* and *Synechococcus*, respectively, based on public data), especially for Sta. 3 and 5, which are closer to BATS (52.0 and 37.4 × 10^3 cell mL^-1 for *Prochlorococcus* and 9.2 and 7.3 × 10^3 cell mL^-1 for *Synechococcus*, respectively). The ~10-fold decrease in *Prochlorococcus* abundances from north to south was also observed by Popendorf et al. (2011) along the same transect (cruise BV39). NP-Euk followed the same trend as picocyanobacteria (0.6 ± 0.3 × 10^3 cell mL^-1, n = 7), whereas populations of P-Euk were more stable across sampled stations (0.7 ± 0.1 × 10^3 cell mL^-1, n = 7). Flow cytometric determinations of cell size based on beads-calibrated-FSC (Casey et al. 2009) indicated that *Prochlorococcus*, *Synechococcus*, and P-Euk were 0.46 ± 0.01, 0.62 ± 0.03, and 1.14 ± 0.05 μm in diameter (Supporting Information Fig. S1), which is smaller than typical culture isolates but comparable to values previously measured in natural populations of the north Atlantic and Pacific gyres (Grob et al. 2013; Rii et al. 2016b). Using cellular carbon quotas measured in the same study area (60, 276, and 14,424 fg C cell^-1 for *Prochlorococcus*, *Synechococcus*, and P-Euk, respectively; Baer et al. 2017), P-Euk represented the large majority of the small phytoplankton (< 5 μm) C biomass (81% ± 10%, 9.5 ± 1.3 μg C L^-1) and *Prochlorococcus* and *Synechococcus* contributed to a similar amount of C biomass (1.1 ± 1.1 and 1.4 ± 0.7 μg C L^-1 or 8% ± 7% and 11% ± 3% of the total small phytoplankton C biomass). Using values from Hartmann et al. (2014) for the Atlantic Ocean (24, 95, and 1978 fg C cell^-1 for *Prochlorococcus*, *Synechococcus*, and P-Euk, respectively), P-Euk would still represent the majority of the small phytoplankton C biomass at 63% ± 15%, whereas *Prochlorococcus* and *Synechococcus* contribution would be 17% ± 12% and 20% ± 4%, respectively (1.3 ± 0.2, 0.4 ± 0.5, and 0.5 ± 0.2 μg C L^-1, respectively).

As a broader context for the research on the nanoplanckton and picoplankton (i.e., small phytoplankton) in the western subtropical North Atlantic samples, we also analyzed the microphytoplankton in four samples taken at Sta. 6, 8, 11, and 14 along the study transect. A broad representation of phytoplankton morpho-groups were observed (Supporting Information Fig. S2), including coccolithophores, diatoms, dinoflagellates, dinoflagellate cysts, and small green-P-Euk. Across the four sampling stations, the range in size varied from 5 to 50 μm, with the highest percentages in the 10 to 20 μm range (Supporting Information Fig. S3). However, the percentages are based on relative abundances observable by light microscopy; and the decrease in percentages below 15 μm may be attributed partially to increasingly fewer cells that were detected by the microscopic techniques. The abundances of total phytoplankton morpho-groups varied from 0.30 to 3.10 × 10^3 cells L^-1, with the highest densities at Sta. 14 near Puerto Rico (Table 2). The lowest abundance was from Sta. 11 where there were also the lowest abundances of *Prochlorococcus*, *Synechococcus*, and some of the lowest P-Euk abundances, in general (Table 2). Because dinoflagellates were observed so frequently across all four stations, their abundances are also reported separately, with a range of 0.3 to 0.5 × 10^3 cells L^-1 and accounting for as much as 30% of the total observed microphytoplankton (Sta. 6; Table 2). Dinoflagellates also accounted
for ~15% of the total phytoplankton counted in Sta. 11 and 14 but only 8% of total phytoplankton in Sta. 8.

**Taxonomic diversity of small-sized protists (< 5 μm) by environmental DNA sequencing**

Molecular sequencing of the sorted cell populations revealed the taxonomic nature of the small-sized eukaryotic assemblages investigated in this study. Although samples were filtered through 8 μm, flow cytometry revealed that sorted cells were essentially <5 μm (Supporting Information Fig. S1). 18S rDNA V4 and/or V8-9 sequences were obtained from Sta. 6, 10, 11, and 14 for two cytometrically sorted (P-Euk and NP-Euk) cell populations. While some OTUs were annotated to the genus level, others could not be, because of the divergence of the reads from the references or because the conserved nature of the 18S V4 or V8-9 regions did not distinguish closely related genera. The two markers yielded similar and complementary results in terms of overall taxonomic composition, although those results differed in some specific details.

For P-Euk, green algae and members of the Dinophyceae (or core dinoflagellates) dominated all the surveyed samples, making up greater than 74% of the analyzed OTUs (or 64% of the total reads; Supporting Information Data S1). Within green algae, OTUs representing the prasinophyte clade IX (Tragin et al. 2016) were most abundant; especially for the Sta. 6 P-Euk sample, this green algal lineage comprised 53% (V4) or 69% (V8-9) of the curated read sets (or 48% or 59% of the total reads). Their cell abundance may surpass the estimates based on read abundance if we consider the possibility that dinoflagellates, including marine alveolates (MALVs), are overrepresented in DNA-based data (as opposed to RNA-based data) due to their high copy number of ribosomal RNA gene cluster, as has been noted previously (Not et al. 2009; Giner et al. 2016). OTUs assigned to MALVs and cercozoans were also present across all P-Euk samples and made up a notable portion of the reads. Detection of nonphotosynthetic MALVs in the pigmented samples may at first glance appear unexpected; however, members of MALVs such as *Ameboflagyr* are parasites of dinoflagellates, including *Gonyaulax* sp. (Kim et al. 2008). Thus, these OTUs may represent those MALVs parasitizing, and thus residing within, a cell of pigmented dinoflagellates. Haptophyte and oocystophyte (i.e., plastid-bearing stramenopiles) OTUs were documented for some, but not all samples. Although diatoms represent only a marginal component of our read data, diatoms that are >10 μm in size were observed from the surveyed samples by microscopy (Table 2; Supporting Information Fig. S2). The relatively low diatom abundances reported here are in the range of values previously published for the Sargasso Sea (Hulburt et al. 1960) and are consistent with biogenic silica concentrations—a proxy for diatom abundance—of the studied waters, which have been suggested to be the lowest in the global surface ocean (Krause et al. 2009). In addition to photosynthetic chlorarachniophytes, heterotrophic cercozoans, including members of *Cryomonadida*, were consistently detected across our sorted P-Euk samples. Chlorophyll signal from these cercozoans may be due to ingested algal prey or algal symbionts. The former scenario is supported by previous studies that showed the cryomonad *Cryothecomonas* spp. feed on large diatoms (Schnepf and Kühn 2000).

Read data for NP-Euk were dominated by MALVs, representing the vast majority (87–100%) of the curated read sets (Supporting Information Data S1). Other OTUs detected from the NP-Euk samples were classified to the cercozoans (e.g., *Minorisa*), stramenopiles such as MAST and labrynthulomyctenes, dinoflagellates, amebozoans, or the radiolarians *Eucyrtidium*, but their collective read abundance was marginal compared to that for MALVs (Fig. 2).

**CO₂ fixation and PO₄ assimilation rates**

Total CO₂ fixation rates (i.e., >0.2 μm size fraction) were fairly uniform along the transect (2.4±0.7 mg C m⁻³ d⁻¹, n = 10; Table 1). These rates were at the lower end of those measured in September at BATS in the first 20 m since 2006 (ranging from 2.0 to 6.0 mg C m⁻³ d⁻¹ and averaging 4.2±1.5 mg C m⁻³ d⁻¹, n = 9, using public data with values below the detection limit discarded). But CO₂ fixation at Sta. 5 (30°40'N 64°19'W), located closest to BATS (31°40'N 64°10'W), was 3.6 mg C m⁻³ d⁻¹. Cell-specific CO₂ fixation rates by *Prochlorococcus* varied from 0.9 to 1.6 fg C cell⁻¹ h⁻¹ (1.1±0.2 fg C cell⁻¹ h⁻¹, n = 10), whereas those by *Synechococcus* were higher and varied from 2.6 to 8.0 fg C cell⁻¹ h⁻¹ (6.1±1.9 fg C cell⁻¹ h⁻¹, n = 10; Fig. 3A). P-Euk presented rates roughly an order of magnitude higher than cyanobacteria (varying from 57.2 to 99.4 fg C cell⁻¹ h⁻¹, averaging 78.9±13.4 fg C cell⁻¹ h⁻¹, n = 10). Within the pigmented protist population, P-Euk_A fixed roughly three times less CO₂ than P-Euk_B (40.3±4.7 and 113.5±25.0 fg C cell⁻¹ h⁻¹, respectively). Those rates were of the same order of magnitude to those measured by Jardillier et al. (2010) over a wider area of the tropical North Atlantic (1.2±0.6, 9.5±4.3, 98.0±50.0, 54.3±19.0, and 230.1±86.1 fg C cell⁻¹ h⁻¹ for *Prochlorococcus*, *Synechococcus*, P-Euk, P-Euk_A, and P-Euk_B, respectively), where CO₂ fixation rates by the whole microbial community was 6.5±3.0 mg C m⁻³ d⁻¹. Using our cell-size estimates and considering a spherical cell volume, biovolume-normalized CO₂ fixation rates were 22±4, 49±17, and 101±11 fg C μm⁻³ h⁻¹ for *Prochlorococcus*, *Synechococcus*, and P-Euk, respectively. This result indicates that P-Euk’s contribution to primary production may not only be due to their larger size but also to higher CO₂ fixation rates per biovolume compared to cyanobacteria. However, using biovolumes from Hartmann et al. (2014), we did not find significant differences between biovolume-normalized CO₂ fixation rates of P-Euk and *Prochlorococcus* (8.0±0.8 and 8.8±1.8 fg C μm⁻³ h⁻¹, respectively), whereas that of *Synechococcus* was higher than for P-Euk (13.6±4.7 fg C μm⁻³ h⁻¹, p < 0.05, two-way analysis of variance multiple comparisons). Using cell abundances, we calculated group-specific volumetric CO₂ fixation rates which
were 28.7 ± 26.4, 31.8 ± 17.6, and 61.4 ± 12.3 ng C L⁻¹ h⁻¹ on average (n = 10) for Prochlorococcus, Synechococcus, and P-Euk, respectively (Fig. 3B).

Total PO₄ uptake rates (i.e., > 0.2 μm size fraction) varied from 0.10 ± 0.00 to 0.33 ± 0.02 nmol L⁻¹ h⁻¹ (mean value: 0.21 ± 0.09 nmol L⁻¹ h⁻¹, n = 15) and were 3.9 ± 1.2-fold lower than the maximum velocity (V_max) measured at a saturating PO₄ concentration (Table 3). Kₘ was low, 3.6 ± 1.1 nmol L⁻¹, possibly indicating that microorganisms were adapted to the PO₄ concentrations measured along the transect. Cell-specific PO₄ uptake rates by Prochlorococcus and Synechococcus (0.37 ± 0.21 and 0.77 ± 0.38 amol cell⁻¹ h⁻¹, respectively, n = 15) were generally lower than by P-Euk and NP-Euk (1.49 ± 0.77 and 1.09 ± 0.46 amol cell⁻¹ h⁻¹, respectively, n = 15; Fig. 4A). However, when normalizing to their respective biovolume, PO₄ uptake by Prochlorococcus and Synechococcus was ~ 4 ± 1 times larger than by P-Euk (7.2 ± 4.3, 6.2 ± 3.2, and 1.9 ± 1.0 amol μm⁻³ h⁻¹, respectively, n = 15). These differences in physiological capacity could indicate more efficient systems for PO₄ acquisition in cyanobacteria than protists in their adaptation to P-limited environments (Casey et al. 2009). Therefore, we investigated small phytoplankton group-specific physiological capacity to acquire PO₄ by measuring their kinetic parameters for PO₄ uptake at Sta. 3 (Fig. 5). Prochlorococcus, Synechococcus, and P-Euk presented a V_max of 12 ± 2, 63 ± 14, and 185 ± 54 amol cell⁻¹ h⁻¹, respectively, and a Kₘ of 11 ± 7, 25 ± 12, and 57 ± 21 nmol L⁻¹, which are in the range of values in Lomas et al. (2014). Group-specific volumetric PO₄ uptake rates were 10.4 ± 11.0, 4.7 ± 3.9, and 1.1 ± 0.6 pmol L⁻¹ h⁻¹ on average (n = 10) for Prochlorococcus, Synechococcus, and P-Euk, respectively (Fig. 4B).

Table 3. PO₄ uptake rates (nmol P L⁻¹ h⁻¹) and Michaelis-Menten kinetic parameters V_max (nmol P L⁻¹ h⁻¹) and K_m (nmol P L⁻¹) in whole samples.

| Sta. | PO₄ uptake rates (nmol P L⁻¹ h⁻¹) | V_max (nmol P L⁻¹ h⁻¹) | K_m (nmol P L⁻¹) |
|------|---------------------------------|-------------------------|------------------|
| 3    | 0.23 ± 0.00                     | 0.94 ± 0.08             | 5.2 ± 2.0        |
| 5    | 0.33 ± 0.02                     | 0.86 ± 0.16             | 3.9 ± 2.9        |
| 8    | 0.29 ± 0.02                     | 0.77 ± 0.07             | 3.7 ± 1.5        |
| 11   | 0.10 ± 0.00                     | 0.55 ± 0.04             | 2.6 ± 1.1        |
| 14   | 0.12 ± 0.00                     | 0.54 ± 0.04             | 2.4 ± 1.0        |

Discussion

P-Euk have a fundamental role in the carbon cycling of the PO₄-deplete western subtropical North Atlantic Ocean

Ocean stratification is predicted to intensify as a result of global warming, with important consequences on nutrient supplies to the upper euphotic zone (Capotondi et al. 2012; Moore et al. 2013). Therefore, understanding marine microbes' response to nutrient depletion is of paramount importance to predict future biogeochemical dynamics in the open ocean. We sampled the western subtropical North Atlantic Ocean during the period of intense water column stratification, which has been shown to induce nutrient depletion and particularly intense PO₄-stress in surface waters (Casey et al. 2009; Lomas et al. 2010). Several parameters confirm PO₄-stress at our sampling sites: PO₄ concentrations were low, the turnover time of PO₄ (T_P04) was short (Table 1), PO₄ uptake kinetic parameters were typical of PO₄-depleted environments (high V_max and low K_m; Table 3), and APA, an indicator of P-stress/limitation, was relatively high.

Bacterivory by small-sized pigmented protists

To obtain an initial estimate of the effects of phosphate concentration on feeding rates, three measurements were made each with an experimental and a control flask. Rates of bacterivory by P-Euk in the control were 0.9 ± 0.3 bacteria P-Euk⁻¹ h⁻¹, at the three stations tested (Fig. 6). Interestingly, rates were reduced to 0.6 ± 0.2 bacteria P-Euk⁻¹ h⁻¹ when a saturating concentration of PO₄ was added in the seawater, indicating potential regulation of feeding rates by PO₄ availability. Because the N is small, we did not do a statistical analysis of the results.

Fig. 3. Box and whisker plots of: (A) cell-specific CO₂ fixation rates (fg C cell⁻¹ h⁻¹) and (B) group-specific volumetric CO₂ fixation rates (ng C L⁻¹ h⁻¹) by Prochlorococcus, Synechococcus, small-sized P-Euk, and small-sized P-Euk subgroups A and B (Pro, Syn, P-Euk, P-Euk_A, and P-Euk_B, respectively) or by the total community. The box extends from the 25th to 75th percentiles. The line in the middle of the box is plotted at the median, whereas the “+” is the mean. The whiskers extend down to the smallest value and up to the largest. Individual values per station are represented by different symbols listed in the right-hand margin (duplicate incubations per station).
Mahaffey et al. (2014) estimated that APA increases significantly when ambient PO$_4$ is below ~ 30 nmol L$^{-1}$. Here, PO$_4$ concentrations at 20 m were < 3 nmol L$^{-1}$, and daily APA rates were 38.4–55.2 nmol L$^{-1}$ d$^{-1}$, which is in the range of values published for the Sargasso Sea but generally higher than APA values measured in the oligotrophic but PO$_4$-replete Pacific Ocean (see review in Mahaffey et al. 2014).

Microbial abundances were largely dominated by pico-cyanobacteria, with Prochlorococcus and Synechococcus representing on average 65% ± 18% and 29% ± 15% of the total small phytoplankton (Prochlorococcus + Synechococcus + P-Euk) cell abundances. However, although P-Euk represented only 5% ± 4% of the small phytoplankton cell abundance on average, this group represented 63% ± 15% to 81% ± 10% of the small phytoplankton C biomass (depending on selected conversion factors) and 53% ± 13% of the volumetric CO$_2$ fixation (ng C L$^{-1}$ h$^{-1}$) by small phytoplankton or 28% ± 6% of the total (> 0.2 μm) primary production (Fig. 3B). Therefore, despite their low abundance, P-Euk—represented mainly by prasinophyte green algae and dinoflagellates (Fig. 2)—have a substantial role in C cycling of the Northwest Atlantic Ocean. Similar results have been found in the North and South Pacific and Atlantic gyres (Jardillier et al. 2010; Grob et al. 2011; Rii et al. 2016a, b). The important role of P-Euk in the oligotrophic ocean C cycling is likely due to their larger cell biovolume relative to picocyanobacteria. Indeed, on a per cell level, P-Euk presented CO$_2$ fixation rates roughly an order of magnitude higher than picocyanobacteria and this relationship held after normalizing to biovolume, although results strongly depended on cell size estimates. Actually, data from the literature vary widely, and cell size estimates obtained using flow cytometry are to be taken with caution (Duhamel et al. 2007; Tzur et al. 2011). Using calibration beads, our estimates indicate that the populations of protists sorted here were smaller than 5 μm (Supporting Information Fig. S1). The isometric scaling of phytoplankton photosynthesis with cell size was first demonstrated using size fractionated measurements of CO$_2$ fixation rates (Marañón et al. 2007; Duhamel and Moutin 2009) and later verified using measurements in flow-cytometry–sorted groups of phytoplankton similar to this study (Jardillier et al. 2010; Grob et al. 2011; Rii et al. 2016a, b). Calculated biomass-normalized
rates of CO₂ fixation, a proxy for growth rates (Marañón 2005; Duhamel et al. 2007), using conversion factors in Hartmann et al. (2014), were 0.56 ± 0.11, 0.78 ± 0.27, 0.58 ± 0.06, and 0.44 ± 0.08 d⁻¹ for Prochlorococcus, Synechococcus, P-Euk_A, and P-Euk_B, respectively. Although comparable to values previously reported (Jardillier et al. 2010; Rii et al. 2016a, b), these estimates should be taken with caution as they are highly dependent on volume-specific C conversion factors and equations that are used to calculate biovolume and C content (Duhamel et al. 2007), particularly considering the much wider size range for P-Euk compared to picocyanobacteria. Nevertheless, this approximation indicates that despite multiple sources of evidence for PO₄-stress, P-Euk could grow roughly as fast as the smaller picocyanobacteria, notwithstanding their potential disadvantage for PO₄ assimilation due to their lower surface to biovolume ratio.

**Low contribution of small eukaryotes to total microbial PO₄ uptake in the PO₄-deplete western subtropical North Atlantic Ocean**

Despite being sizable, the contribution of P-Euk and NP-Euk to the summed small plankton (Prochlorococcus + Synechococcus + P-Euk + NP-Euk) volumetric PO₄ assimilation (nmol L⁻¹ h⁻¹; Fig. 4B) was low (11% ± 8% and 8% ± 5%, respectively) in comparison to Prochlorococcus and Synechococcus (50% ± 20% and 32% ± 9%, respectively). Although the contribution of heterotrophic bacteria was not assessed in this study, this group often dominates volumetric uptake of PO₄ by the total microbial community (Michelou et al. 2011; Duhamel et al. 2014; Popendorf and Duhamel 2015) and therefore percent values listed above would be much reduced if heterotrophic bacteria were included. In fact, on average, small eukaryotes represented less than ~1% of the total uptake of PO₄ (> 0.2 μm), equivalent to previous findings in the oligotrophic North Atlantic (Casey et al. 2009; Hartmann et al. 2011). The low contribution of small eukaryotes to total microbial PO₄ uptake is a result of their lower abundance, but also due to their low biovolume-normalized assimilation rates compared to bacteria and cyanobacteria (Popendorf and Duhamel 2015). In the present study, we found that cell-specific PO₄ uptake rates by cyanobacteria were generally lower than by small eukaryote groups, but when normalizing to their respective biovolume, PO₄ uptake by cyanobacteria was ~4 ± 1 times larger than by P-Euk. In the PO₄-depleted Gulf of Mexico, Popendorf and Duhamel (2015) found that on a per-cell basis, P-Euk presented the highest PO₄ assimilation rates, but biovolume-normalized heterotrophic bacteria PO₄ assimilation rates were roughly an order of magnitude higher than those of cyanobacteria and P-Euk. Similarly, in the North Atlantic subtropical gyre, Hartmann et al. (2011) found that the biomass-specific PO₄ uptake rates of small eukaryotes (P-Euk and NP-Euk) were only 5–15% of the biomass-specific rates of bacterioplankton. Therefore, P-Euk have high cell-specific PO₄ uptake rates but low volume-specific PO₄ uptake rates, compared with cyanobacteria.

As a result of their contrasted contribution to CO₂ and PO₄ assimilation rates, on average, P-Euk presented much higher cell-specific C : P uptake ratios than picocyanobacteria (144 ± 74, 332 ± 137, and 2695 ± 2046 for Prochlorococcus, Synechococcus, and P-Euk, respectively; Fig. 7). This ratio tended to be higher in the most southern stations (11 and 14), mostly as the result of decrease in PO₄ uptake, indicating sustained primary productivity despite reduced PO₄ availability. Even higher C : P assimilation ratios by P-Euk have been estimated in the central North Atlantic (2000–4000) by Hartmann et al. (2011) based on their PO₄ uptake measurements and parallel CO₂ fixation measurements by Grob et al. (2011). These much higher C : P uptake ratios indicate that P-Euk may respond to low PO₄ availability differently than picocyanobacteria.

**How do small protists cope with low PO₄ availability in the Northwest Atlantic Ocean?**

The relatively low biovolume-normalized PO₄ assimilation by small protists and high C : P uptake ratio for P-Euk can be explained by several nonexclusive scenarios. Small protists may reduce their cellular P requirements by altering their biochemical composition and/or relying on alternative PO₄ acquisition pathway(s). To reduce their P requirements, phytoplankton groups can substitute non-P (sulfur- and nitrogen-containing) lipids for phospholipids in their membranes under low-PO₄ conditions (Van Mooy et al. 2009). Observations in controlled laboratory cultures revealed that eukaryotic phytoplankton lipid content may change rapidly with short-term variations in PO₄ supply, indicating that lipid remodeling is not simply a result of long-term adaptation to the environment (Martin et al. 2011). Another possibility for phytoplankton to reduce their PO₄ uptake needs would be to use their polyphosphate reserves, a molecule thought to serve as
luxury PO₄ storage. However, this view has been recently challenged when P-stressed phytoplankton from the PO₄-depleted Sargasso Sea were found to be enriched in polyphosphate compared with nutrient-rich temperate waters (Martin et al. 2014). Overall, these alterations of the biochemical composition of phytoplankton are difficult to quantify on a group-specific level, especially for low abundant plankton such as protists (Popendorf and Duhamel 2015) and in particular, for polyphosphate concentrations that presently is still semiquantitative (Martin and Van Mooy 2013).

Our APA data suggest that utilization of dissolved organic phosphorus (DOP) may be one of the alternative PO₄ acquisition pathways (about a third of APA was measured in the fraction > 0.8 μm). However, it is unclear what organic molecules may be utilized. Using ATP as a proxy for DOP, we have shown previously that P-Euk in surface samples from the oligotrophic Gulf of Mexico could assimilate P from ATP, but P uptake rate per biovolume from ATP represented less than 5% compared to uptake from PO₄. Therefore, small-sized protists may rely primarily on PO₄ for osmotrophy. PO₄ uptake kinetic parameters indicated that P-Euk could potentially increase their V_max by two orders of magnitude compared to uptake at ambient low-PO₄ concentrations, but their K_m was much higher than picocyanobacteria (Fig. 5). Based on the theory of diffusion limitation, uptake affinity should scale linearly with the cell radius, and for marine species, affinity normalized by the minimum P quotas is expected to increase with decreasing cell size (Edwards et al. 2012). Plotting our limited dataset of estimated group-specific cell size and biomass-specific affinity (α, the ratio of V_max/Qₚ to K_m, with Qₚ the P quotas based on median values measured in the Northwest Atlantic in Baer et al. [2017]) revealed a linear inverse relationship (α [L nmol P⁻¹ h⁻¹] = −0.042 × cell size [μm] + 0.053, R² = 0.958). Therefore, our results support previous findings based on data from laboratory cultures, that marine phytoplankton ability to compete for limiting PO₄ tends to increase with decreasing cell size (Edwards et al. 2012). However, a recent study suggests that although small cells are indeed favored by a large surface-to-volume ratio, they suffer from needing a higher transporter density to maximize their affinity and from a higher relative cost of a transporter (Lindemann et al. 2016).

Predation on protskaryotes (heterotrophic bacteria and picocyanobacteria) may be another pathway for small protists, both heterotrophic and mixotrophic, to acquire additional PO₄. In the central North Atlantic Ocean, P-Euk and NP-Euk have been shown to consume similar quantities of bacterioplankton (Zubkov and Tarran 2008). Protists may even be able to select their prey, favoring certain sizes, shapes, species, and elemental composition (Pernthaler 2005). Previous studies using protists in culture showed that biochemical variations within prey species may influence predator species composition, abundance, feeding rates, and elemental composition (Boeningk et al. 2001; Pernthaler 2005; Meunier et al. 2012; Zhang et al. 2017). As bacteria biomass-normalized PO₄ assimilation is much greater than protists (Casey et al. 2009; Hartmann et al. 2011; Popendorf and Duhamel 2015), the capacity to consume this relatively P-rich food would represent a competitive advantage for protists that could compensate for their low biomass-specific affinity in comparison to picocyanobacteria. Considering our measured rates of bacterivory by P-Euk (0.9 ± 0.3 bacteria protist⁻¹ h⁻¹) and estimated values of Qₚ, of bacteria at BATS (4.0–6.4 amol P cell⁻¹ for bacteria 0.025–0.042 μm³; Gundersen et al. 2002), we calculated that P-Euk could obtain ~ 4–6 amol P cell⁻¹ h⁻¹ through phagocytosis, which is ~ 3–4 times higher than the median value for PO₄ assimilation rates by osmotrophy in our dataset. Phagocytosis would therefore allow P-Euk to replace nearly 15% of their Qₚ each day (Qₚ of P-Euk in the Sargasso Sea is 900 amol P cell⁻¹; Baer et al. 2017). This is of course a rough estimate considering our conservative bacterivory rates, the variable Qₚ of heterotrophic bacteria, and the fact that ingested cells are partly excreted and therefore not fully metabolized, but it underlines the undeniable advantage for a small protist to ingest prokaryotes to acquire nutrients. Interestingly, previous findings from laboratory culture experiments indicate that under PO₄-limited conditions, mixotrophs typically retain most of the P acquired through bacterial ingestion; in contrast to exclusive phagotrophs, which release P during grazing (Rothhaupt 1997). Therefore, mixotrophs would not contribute to promoting the growth of competing phytoplankton (Stukel et al. 2011).

Molecular sequencing of the P-Euk confirmed the presence of several taxonomic groups known to be mixotrophic. In particular, most of the P-Euk samples were represented by three or more OTUs assigned to the core dinoflagellates. This is consistent with our evidence obtained by light microscopic enumeration of microphytoplankton in four stations along the transect, where dinoflagellates accounted for up to 30% of the total microphytoplankton. Of the dinoflagellate OTUs, some were assigned to Azadinium, Amphidoma, and Gonyaulax; most, if not all, members of which have been suggested to be mixotrophic (Stoecker 1999). Green algae, particularly those that fall within the prasinophyte clade IX, represented a notable fraction, ranging from 8% to 70% of the curated reads for the P-Euk samples. Prasinophyte IX is a green algal lineage that was first recognized from surface ocean areas in the Mediterranean Sea samples (Viprey et al. 2008) and then from a number of other ocean sites, particularly oligotrophic waters (Shi et al. 2009). A culturable representative from the lineage has not yet been established. Besides their propensity for warm, low-nutrient seawaters and their pico-sized range (Shi et al. 2009; Tragin and Vaulot 2018), not much is known about their biology, including nutritional mode (e.g., presence or absence of bacterivory).

**PO₄-regulated bacterivory by small mixotrophic eukaryotes**

Rates of bacterivory by P-Euk were on average less than 1 bacteria P-Euk⁻¹ h⁻¹, which is well within the range of values previously reported for pigmented nanoflagellates.
measured using fluorescently labeled bacteria (Havskum and Riemann 1996; Christaki et al. 1999; Unrein et al. 2014) and for P-Euk groups measured using the technique used here (Zubkov and Tarran 2008; Hartmann et al. 2012). Still, the approach employed here likely underestimates rates by the small mixotrophic eukaryotes as it represents an average for the total P-Euk population, which includes strictly autotrophic organisms, mixotrophic organisms not actively feeding, and mixotrophic organisms feeding at different time of the day (Anderson et al. 2017). Furthermore, we cannot exclude that some of the 35S tracer could be released by protists after 35S-smethionine metabolism (Zubkov and Tarran 2008). Interestingly, the addition of PO4 in the seawater samples resulted in 32% ± 5% decrease in bacterivory rates by P-Euk, indicating that prey ingestion rates by small-sized mixotrophic eukaryotes may be regulated by ambient nutrient concentration. Similar results were obtained by Christaki et al. (1999) and Arenovski et al. (1995) using nutrient amendment assays with PO4-depleted Mediterranean and Sargasso seawater, respectively. The authors found that while treatments including PO4 resulted in increased bacterial production, the number of mixotrophs identifiable using fluorescent food tracer particles was reduced. Interestingly, regulation of phagotrophy by nutrient availability may only happen in the case of PO4 stress for the mixotrophic green alga Cymbomonas tetraniiformis (A. Paasch et al. unpubl.). Bacterivory by small mixotrophic protists may be important to alleviate PO4 stress and may therefore sustain primary production in the PO4-depleted western subtropical North Atlantic Ocean. Unfortunately, our attempt to quantify the fraction of active bacterivores among P-Euk using prey labeled with pHrodo Green Dye with detection by flow cytometry (First et al. 2012) failed due to the very low number of detected P-Euk showing increased green fluorescence. However, this result may have been due to technical challenges with using this method on samples with low protists abundance and/or on fixed samples (Sieracki et al. 1987; N. Bock et al. unpubl.). In a previous study at a similar location, Arenovski et al. (1995) found that up to 50% of the pigmented nano-sized eukaryotes in surface waters ingested fluorescent tracers, but smaller protists (picoeukaryotes) were not studied. Nonetheless, based on 18S rDNA amplicon sequence data from the total P-Euk fraction and published knowledge on the nutritional mode of annotated eukaryotic groups, we infer that bacterivores among P-Euk in our surveyed samples include mixotrophic members of dinoflagellates (e.g., Azadinium) and chlorarachniophytes as well as heterotrophic cercozoan flagellates.

Conclusion

We found that despite their low abundance, P-Euk have a substantial role in the C cycling of the western subtropical North Atlantic Ocean: they represented roughly two thirds of the microbial phytoplankton C biomass and half of the volumetric CO2 fixation by the microbial phytoplankton or a third of the total primary production. However, in the PO4-depleted surface waters, P-Euk and NP-Euk represented each roughly 10% of the summed microbial plankton volumetric PO4 assimilation and much less if bacterioplankton were considered. This resulted in unusually high C : P uptake ratios, especially compared to picocyanobacteria. In view of the relatively low biovolume-normalized PO4 assimilation by small protists, the high C : P uptake ratio may be explained by several nonexclusive scenarios, but a back-of-the-envelope calculation indicates that predation on relatively P-rich prokaryotes may provide both mixotrophic and strictly heterotrophic protists with their largest source of PO4. Although phagotrophy may be a competitive advantage for mixotrophic protists during extended periods of PO4 limitation in the western subtropical North Atlantic Ocean, this is a costly PO4 acquisition pathway to maintain for a phagotrophic protist that is also osmotrophic (Raven 1997). That would explain the observed decrease in bacterivory rates when PO4 was added in the seawater. Our results show that small-sized, pigmented protists of the western subtropical North Atlantic Ocean are dominated by members of dinoflagellates, prasinophycean green algae, and cercozoans, with a major role in the cycling of C, nutrients, and organic matter. Shifts in species composition and in the relative fraction of mixotrophic vs. strictly autotrophic P-Euk could potentially have a sizable impact on the biogeochemical cycles of the oligotrophic oceans. Prospective research investigating the drivers of mixotrophy by small-sized protists will be key to predicting microbial composition and biochemical state in a changing ocean.

Author Contribution Statement:

S.D., E.K., and O.R.A. contributed to the planning and project development and provided data. S.D. and E.K. conducted field work. S.D., E.K., B.S., and O.R.A. contributed to data analyses. S.D. authored/drafted this manuscript. All authors provided intellectual content, revised, and provided final approval and are accountable for this manuscript.

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Conflict of Interest

None declared.

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