Mixotrophic basis of Atlantic oligotrophic ecosystems

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Oligotrophic subtropical gyres are the largest oceanic ecosystems, covering >40% of the Earth’s surface. Uncellular cyanobacteria and the smallest algae (plastic protists) dominate CO2 fixation in these ecosystems, competing for dissolved inorganic nutrients. Here we present direct evidence from the surface mixed layer of the subtropical gyres and adjacent equatorial and temperate regions of the Atlantic Ocean, collected on three Atlantic Meridional Transect cruises on consecutive years, that bacterioplankton are fed on by plastidic and aplastidic protists at comparable rates. Rates of bacterivory were similar in the light and dark. Furthermore, because of their higher abundance, it is the plastidic protists, rather than the aplastidic forms, that control bacterivory in these waters. These findings change our basic understanding of food web function in the open ocean, because plastidic protists should now be considered as the main bacterivores as well as the main CO2 fixers in the oligotrophic gyres.

phytoplankton predation | phototrophic eukaryotes | primary producers | microbial grazers | pulse-chase labeling

Oligotrophic ecosystems of subtropical oceanic gyres are the most extensive ecosystems on Earth. These ecosystems cover ~40% of the planet’s surface, with their area currently expanding (1). Prochlorococcus cyanobacteria and the SAR11 group of α-proteobacteria are the most numerous microbes in these ecosystems (2, 3), whereas the smallest plastidic protists, comprising various taxonomic groups including uncultured members of the Prymnesiophyceae and Chrysophyceae (4–8), are the most numerous among the eukaryotes, dominating over their aplastidic counterparts (9). The large area of these oligotrophic gyres means that they play a key role in global biogeochemical cycles. However, current knowledge about the functioning of these microbe-controlled systems is relatively limited, owing to the difficulty of studying microbes in a photic layer typified by nanomolar concentrations of inorganic macronutrients.

According to our current understanding of oligotrophic ecosystem functioning, the roles of different microbial populations are tightly defined. In the established paradigm (10) for these systems, phytoplankton such as cyanobacteria and plastidic protists harvest light, fix CO2, and take up inorganic nutrients. They are the primary producers of organic matter that fuels the entire system, allowing heterotrophic bacterioplankton, dominated by the SAR11 group, to thrive. Populations of both cyanobacteria and heterotrophic bacterioplankton are controlled by viruses and aplastid protist predators. Organic matter and inorganic nutrients, released by these control processes, in addition to dead cell death and bacterioplankton remineralization of dissolved organic matter, sustain heterotrophic bacterioplankton and phytoplankton. However, some more recent observations are at variance with this paradigm.

It is generally accepted that prokaryotes are more efficient than protists in acquiring nutrients at low concentration because of their higher cell surface-to-volume ratio (11). Indeed, in the North Atlantic subtropical gyre, bacterioplankton dominate phosphate uptake and outcompete protists for this nutrient (12, 13). However, despite their low phosphate uptake, plastidic protists are major contributors to CO2 fixation (5, 14). Consequently, the C:P ratio, calculated using CO2 and phosphate uptake rates by plastidic protists, is unrealistically high, suggesting that osmotrophic uptake of phosphate cannot satisfy protist requirements for growth (12). Therefore, to sustain themselves in oligotrophic ecosystems, plastidic protists must somehow be able to compensate for a lack of inorganic nutrients. We hypothesize that they do this by mixotrophy: They gain energy from sunlight and simultaneously prey on bacterioplankton to acquire organic, and perhaps some essential organic, nutrients, such as amino acids and vitamins.

Mixotrophy in natural populations of large (>3 μm) plastidic protists has been previously documented microscopically in coastal oligotrophic and upwelling regions as well as in the open equatorial Pacific Ocean and the Mediterranean Sea (15–17). There is also qualitative molecular evidence from the subtropical North Pacific of the presence of mixotrophs among picocyanobacterial predators (18). Furthermore, the quantitative dominance of bacterivory by small plastidic protists (<3 μm) over aplastidic protists has been reported for the temperate North Atlantic Ocean in summer (19). The latter paper also outlines preliminary evidence of bacterivory by plastidic protists in the mesotrophic subtropical northeast Atlantic Ocean. However, the ecological extent of mixotrophy in the vast ecosystems of the oligotrophic open ocean remains unknown.

Here we show that plastidic protists prey on bacterioplankton in the surface mixed layer of both oligotrophic subtropical gyres and adjoining low-latitude pelagic regions of the Atlantic Ocean (40°N to 40°S). Owing to their high abundance, plastidic protists prevail over aplastid protists in bacterivory. This evidence suggests that mixotrophy is crucial to sustain the functioning of oligotrophic marine ecosystems.

Results

Proti bacterivory was assessed on three Atlantic Meridional Transect (AMT) research cruises in October–November 2008, 2009, and 2010 encompassing subtropical oligotrophic gyres of the Northern and Southern hemispheres and the equatorial convergence area (Fig. 1). Temperate waters adjoining the Southern gyre were also examined. The results of an earlier study conducted in 2007 in North Atlantic temperate waters (19) are included for comparison.

Proti feeding on bacterioplankton (Bpl) was determined using a dual-labeling pulse–chase method (20) and flow cytometric sorting of labeled prey and predator cells (19, 21). Three populations of the smallest planktonic protists were examined (Fig. S1): plastidic protists (i.e., chloroplast-containing) smaller, ~2 μm (Plast-S) and larger, ~3 μm (Plast-L) as well as aplastid (without chloroplast) protists, ~3 μm (Aplast) (Table S1). The two size

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PNAS Early Edition | 1 of 5
classes of plastidic protists were operationally differentiated by flow cytometry using cell 90° light scatter, DNA content, and autofluorescence (Fig. S1).

Bacterioplankton cells were labeled using a pulse chase of two amino acids, [35S]methionine and [3H]leucine (Materials and Methods), to examine digestion of prey biomass by protist predators (SI Text). In the majority of experiments, in all regions studied, tracer content of protist cells increased with time during the chase phase in contrast to stable or slightly decreased tracer content of bacterioplankton cells (Figs. S2–S4). The increase demonstrates bacterivory by the three types of protist cells. More robust 35S-based assessments (SI Text) were used to compare protist bacterivory in different oceanic regions (Fig. 2). Because only two stations were examined on each cruise in the Southern temperate (ST) region in austral spring, the 2008, 2009, and 2010 measurements were combined to get a more representative average estimate of protist bacterivory. The rates of bacterivory compared favorably with independent estimates (SI Text) derived from cell-uptake rates of phosphate in surface waters of the Northern subtropical gyre (NG). Moreover, the influence of light and dark incubation on bacterivory was studied on the cruise in 2010. No statistically significant light-induced differences (t test, P > 0.1) in protist bacterivory were determined (Fig. S2, SG 2010).

The rates of cell bacterivory (i.e., the number of bacterioplankton cells consumed per protist cell·h−1) by the Aplast protists were comparable in all regions apart from the Northern temperate (NT) region in summer (Fig. 2). The difference between the ST and the NT regions was probably seasonal. Bacterivory by the Plast-L cells was the lowest in the Southern tropical gyre (SG) in 2008 and 2010 but was comparable to bacterivory by Aplast cells in the ST region. Bacterivory by the Plast-S cells was lowest in temperate waters and in the SG in 2010. It was similar to bacterivory by the Plast-L cells in the NG in 2009 and in the SG in 2008, but lower in the SG in 2009 and 2010.

Rates of bacterivory in the SG varied interannually. Cell bacterivory by all three types of protists was significantly higher in 2009 compared with 2008 and 2010, whereas the differences between 2008 and 2010 were insignificant (Fig. 2). On the other hand, the concentration/biomass of the Plast-S population and the concentration of bacterioplankton were comparable between the 3 y (Fig. 3A and Fig. S5), whereas the concentration/biomass of Aplast and the Plast-L protists was higher in 2010. For comparison, Synechococcus and Prochlorococcus concentrations were 70% and 50% higher in 2010 compared with 2008, respectively (Fig. 1B–D and Fig. S6). Bacterioplankton, Synechococcus, and Prochlorococcus concentrations in the surface mixed layer of the two gyres were similar in 2009, whereas Plast-L and Aplast biomass was lower in the SG than in the NG, and the opposite was true for the Plast-S protists (Fig. 1C and Figs. S5 and S6). The biomass of Plast-L protists was highest in all regions, followed by the biomass of Aplast and Plast-S protists (Fig. 3A and B). The combined biomass of the two plastidic protist groups made up between 65% and 90% of the combined biomass of the smallest protists (Fig. 3B).

In contrast to cell bacterivory, population bacterivory (i.e., the total number of bacterioplankton consumed ml−1·h−1 by each
Discussion

The uniformly higher population rates of bacterivory by plastidic protists compared with aplasticid protists in the surface mixed layer of the Northern and Southern gyres and the equatorial region show the large contribution of phytoplankton to harvesting bacterioplankton in the low-latitude Atlantic Ocean. Furthermore, interannual variability had a minor effect on the domination of bacterivory by plastidic protists.

Fourth Implication

The fourth implication concerns the ecological significance of the smallest plastidic protists in oligotrophic ecosystems. Apart from being key CO$_2$ fixers (5, 14), plastidic protists control bacterioplankton abundance, acting as producers of organic matter and predators at the same time. Such dual control and interdependence of bacterioplankton and protists could help to explain the constancy of low bacterioplankton concentrations in the oligotrophic ocean compared with more productive regions (34, 35). The scarcity of bacterioplankton prey in oligotrophic gyres in turn probably reduces both propagation of phage infections and growth of specialized predators such as aplasticid protists.

Summary

In summary, this work shows the significance and ubiquity of mixotrophy in the survival of the smallest pelagic protists in sunlit oligotrophic surface waters. This deceptively inefficient lifestyle should reduce nutrient export and maintain faster nutrient turnover in the surface mixed layer, both of which are

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\text{Fig. 3. A comparison of mean absolute (A) and relative (B) population biomass and mean absolute (C) and relative (D) population bacterivory of aplasticid, large plastidic, and small plastidic protists in the five regions (see Fig. 1 for details). The numbers next to the region abbreviations indicate the number of experiments done in each region. The rates were calculated using [35S]methionine pulse-chase tracing. Error bars show single SDs to indicate the variance of biomass and rates within regions.}
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This study, comprising 68 experiments, was carried out in the Atlantic Ocean during one AMT cruise on board the UK Royal Research Ship (RRS) James Clark Ross in October–November 2008 and on two AMT cruises on board the UK RRS James Cook in October–November 2009 and 2010 (Fig. 1). Seawater samples were collected from a depth of 20 m before dawn with 20-L Niskin bottles mounted on a sampling rosette of a conductivity-temperature-depth profiler (Sea-Bird Electronics). In 2008 and 2009, experiments were set up within 20 min of sample collection in the dark at ambient temperature, controlled by a water bath to maintain temperature within 0.5 °C. In 2010, experiments were set up for dark and light measurements in a dark room using only a dim green light (LEE filter 090; transmission of 20–30% of light at 500–550 nm). Light incubation experiments were placed in a 6-L water tank illuminated by a warm white light-emitting diode (LED) array (Photon Systems Instruments). Parallel dark incubations were done in a similar 2-L water tank without LED illumination. Filters were mounted onto glass slides and into a refrigerated bath (Grant Instruments) to maintain temperature within 0.5 °C of in situ temperature at the depth of sample collection. The LED array was adjusted to keep light intensity at 300 μmol photons m−2 s−1 inside the incubation bottles.

**Cell Counting.** Bacterioplankton and protist cell concentrations were determined by flow cytometry (Fig. 51) using FACSort and FACSCalibur instruments (BD Biosciences). Synechococcus and Prochlorococcus cyanobacteria were counted in unfixed samples (Fig. 1 B–D and Fig. 56). Concentrations of Prochlorococcus in the surface mixed layer were likely underestimated, owing to low chlorophyll content of cells that led to red auto-fluorescence of cells lying closer to the detection limit of the flow cytometers. The other samples were fixed with paraformaldehyde (PFA) 1% (wt/vol) final concentration and stained with SYBR Green I DNA dye (13, 36). A mixture of multfluorescent beads, of diameter 0.5 μm and 1.0 μm (fluoresbrite microparticles; Polysciences), was used as an internal standard for fluorescence and flow rates (37). To compare protist population biomass (Fig. 3 A and B), protist concentrations were multiplied by the corresponding cell biomass values.

To estimate their cell sizes, the three groups of protists were flow-sorted (Fig. 51), sorted cells being deposited on polycarbonate membrane filters with a 0.2-μm pore diameter. Filters were mounted onto glass slides and stained with DAPI (final concentration 1 μg mL−1) to reveal cell cytoplasm. Aplast cells were sorted and sized from four experimental samples on the 2009 cruise and from five experimental samples on the 2010 cruise, which represented all regions studied. Plast-L and Plast-S cells were sorted from five and four experimental samples on the 2010 cruise, respectively. At least 200 cells were measured at 1,000× magnification of an epifluorescence microscope (Axioskop 2; Zeiss) to estimate mean diameters. Mean cell diameters of each of the three protist groups were statistically similar in analyzed samples (Table 51). The overall mean size of Plast-S cells of 2.0 ± 0.1 μm was significantly lower (t test, P = 0.0002) than the overall mean for Plast-L cells of 3.1 ± 0.3 μm, whereas the overall mean size of Aplast cells of 2.9 ± 0.3 μm was statistically similar to the size of Plast-L cells. To estimate the biomass of the three protist groups, their cell bio-volume was computed assuming that the cells were spheres with a diameter equal to the mean cell size. Cell biovolume was converted into cell biomass using a specific carbon content of 200 fg C μm−3, taken as a mean value from Christian and Karl (38).

**Determining Rates of Protist Bacterivory Using Pulse–Chase Dual Labeling of Natural Communities.** Before an experiment, glass bottles (250 mL Schott; Fisher Scientific) were soaked in 10% hydrochloric acid and rinsed twice with 50% ethanol (twice). Bottles were taken from the same Niskin bottle as that for the subsequent experiment. Seawater (250 mL) from the sample was subsequently added to each washed glass bottle and spiked with [15S]-methylene (specific activity >37 TBq/mmol; Hartmann Analytic), final concentration 0.25 nM or 0.4 nM, and L-(4,5-3H)-leucine (specific activity 1.48–2.22 TBq/mmol; Hartmann Analytic), final concentration 0.5 nM. An increase in the amount of label (and thus the sensitivity of the experiment) was necessary to compensate for the much lower batch size and overall mean cell biomass of Plast-L cells of 3.1 ± 0.3 μm. Four different populations (total bacterioplankton, Plast-L, Aplast-L, and Aplast protists) were sorted (Fig. 51). For each population, four to eight replicates of different cell numbers were sorted (19).

Sorted bacterioplankton cells were collected onto 0.2-μm polycarbonate filters. Sorted protist cells were collected onto 0.8-μm polycarbonate filters. Sorted protist cells were collected onto 0.8-μm polycarbonate filters to reduce the potential of potentially by-sorted Bpl cells. Filters were washed with deionized water to remove contaminating tracer and placed into scintillation vials to which 5 mL of scintillation mixture (Goldstar) was added. Subsequently, the vials were radioassayed for 0.5–2 h (depending on sample radioactivity) using ultra–low-level liquid scintillation counters (1220 Quantulus; Wallac).

**Data Analyses.** Using quench curves, the 3H label was deconvoluted from the 35S label, and the amount of each label was computed as Bq cell−1 by dividing the cumulative 3H or 35S radioactivity by the corresponding number of sorted cells. Cell radioactivities at 3 h and 9 h were compared including dark and light incubations (Fig. S2).

**Cell bacterioly was calculated according to the following formula:**

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\text{Bacterioly} = \frac{\text{Pr}_{\text{t}}}{\text{Pr}_{\text{t}}} \times \frac{\text{Bp}_{\text{t}}}{\text{Bp}_{\text{t}}} \times \left(1 - \frac{\text{Pr}_{\text{t}}}{\text{Pr}_{\text{t}}}ight) \times \left(1 - \frac{\text{Bp}_{\text{t}}}{\text{Bp}_{\text{t}}}ight)
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where \(\text{Pr}_{\text{t}}\) is the average activity of four to eight replicates of one of the protist groups at the second time point and \(\text{Pr}_{\text{t}}\) is the same at the first time point; \(\text{T1} \) and \(\text{T2} \) are the first and second time points of the experiment (e.g., 3 h and 9 h, respectively); \(\text{Bp}_{\text{t}}\) and \(\text{Bp}_{\text{t}}\) are the average activity of four to eight replicates of the bacterioplankton cells at \(\text{T1} \) and \(\text{T2} \), respectively. Because of the pulse–chase experimental design, the activity of the Bpl was in most cases the same at \(\text{T1} \) and \(\text{T2} \) (Figs. S2–S4) and a cumulative mean could be used.

To verify that the increase in label between the first and second time points was statistically significant, t tests (P < 0.05) were carried out using SigmaPlot version 11.0 (Systat Software) and Quattro-Pro X4 (Corel) software. Errors were calculated according to SE propagation procedures. The majority of experiments (80%) showed a significant difference in protist radioactivity between the two time points (Figs. S2 and S4) and hence demonstrated bacterivorous activity of the Plast-S, Plast-L, and Aplast cells. We attribute nonsignificant bacterivory in some experiments to the detection limit of our method owing to the low radioactivities measured. All major radioactivities (80%) were added at concentrations to match the values used in the chase (see above), and in addition radioactivators were added at concentrations identical to the ones used in the pulse phase. Subsamples were fixed for flow sorting after 3 h and 9 h. The measured radioactivity in chase–pulse–labeled and –sorted cells was compared to the background (Fig. S4), confirming the insignificant osmotic gradient of tracer molecules by sorted protists and bacterioplankton during the chase phase.

**Flow Cytometric Cell Sorting.** PFA-fixed radioactively labeled samples were stored at 4 °C and sorted within 10 h. For each time point, four different populations (total bacterioplankton, Plast-S, Plast-L, and Aplast protists) were sorted (Fig. 51). For each population, four to eight replicates of different cell numbers were sorted (19).

Sorted bacterioplankton cells were collected onto 0.2-μm polycarbonate filters. Sorted protist cells were collected onto 0.8-μm polycarbonate filters to reduce the potential of potentially by-sorted Bpl cells. Filters were washed with deionized water to remove contaminating tracer and placed into scintillation vials to which 5 mL of scintillation mixture (Goldstar) was added. Subsequently, the vials were radioassayed for 0.5–2 h (depending on sample radioactivity) using ultra–low-level liquid scintillation counters (1220 Quantulus; Wallac).
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