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Decrease in the Autotrophic-to-Heterotrophic Biomass Ratio of Picoplankton in Oligotrophic Marine Waters Due to Bottle Enclosure▽

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We investigated the effects of bottle enclosure on autotrophic and heterotrophic picoplankton in North and South subtropical Atlantic oligotrophic waters, where the biomass and metabolism of the microbial community are dominated by the picoplankton size class. We measured changes in both autotrophic (*Prochlorococcus*, *Synechococcus*, and picoeukaryotes) and heterotrophic picoplankton biomass during three time series experiments and in 16 endpoint experiments over 24 h in light and dark treatments. Our results showed a divergent effect of bottle incubation on the autotrophic and heterotrophic components of the picoplankton community. The biomass of picophytoplankton showed, on average, a >50% decrease, mostly affecting the picoeukaryotes and, to a lesser extent, *Prochlorococcus*. In contrast, the biomass of heterotrophic bacteria remained constant or increased during the incubations. We also sampled 10 stations during a Lagrangian study in the North Atlantic subtropical gyre, which enabled us to compare the observed changes in the auto- to heterotrophic picoplankton biomass ratio (AB:HB ratio) inside the incubation bottles with those taking place *in situ*. While the AB:HB ratio *in situ* remained fairly constant during the Lagrangian study, it decreased significantly during the 24 h of incubation experiments. Thus, the rapid biomass changes observed in the incubations are artifacts resulting from bottle confinement and do not take place in natural conditions. Our results suggest that short (<1 day) bottle incubations in oligotrophic waters may lead to biased estimates of the microbial metabolic balance by underestimating primary production and/or overestimating bacterial respiration.

Seawater confinement is often used to study the dynamics and metabolic rates of microbial plankton communities. Small water volumes (usually not larger than 200 ml) and incubation times typically up to 24 h are frequently used to measure community respiration rates and bacterial and primary production. The key assumption of these experiments with bottle incubations is that they adequately represent the measured rates, thus allowing their extrapolation to *in situ* communities. Ideally, the *in situ* community structure should be reflected in the initial community inside the bottle, but larger cells such as micro- and nanoplanktonic protists and copepods are more easily underrepresented in small volumes (2), which can potentially cause alterations in the trophic interactions taking place within the community (35). Furthermore, confinement prevents exchange of nutrients and metabolites with surrounding water. All these effects may either stimulate the growth or enhance the loss processes of different planktonic functional groups (8), which has been referred to as the "bottle enclosure effect" (12).

Previous studies have shown a significant change in phylogenetic composition (22) as well as an increase of abundance (5, 26), culturability (7), and metabolic activity (30) of marine heterotrophic bacteria during bottle incubations of untreated water samples. Examples of detrimental effects of bottle incubations on autotrophic components can also be found in the literature. Gieskes et al. (12) and Harris (14) found high rates of photochemical pigment destruction when enclosing oligotrophic water in small bottles and lower cell abundance and primary production rates compared with estimates obtained in larger bottles with the same oligotrophic water samples. Large short-term changes (<6 to 7 h) in the biomass of picophytoplankton (*Prochlorococcus*, *Synechococcus*, and eukaryotes) were observed by Fernández et al. (8) in the subtropical North Atlantic, with an up to 75% decrease in *Prochlorococcus* biomass after only 2.5 h. Hence, it seems that there is a general detrimental effect on phytoplankton and an enhancement effect on heterotrophic bacterioplankton during bottle incubations. However, as far as we know, there are no reports assessing bottle enclosure effects simultaneously on both auto- and heterotrophic components of microbial plankton in unamended oligotrophic marine waters. This is highly relevant if data derived from bottle incubations are used to estimate the metabolic balance between production and respiration, resulting in biogeochemical conclusions.

There is still uncertainty on whether the large oligotrophic regions in the oceans, overwhelmingly dominated by the small organisms in the picoplankton size class (1, 18, 20), are net autotrophic or net heterotrophic. Studies derived from indirect and direct geochemical approaches, with long integration timescales, systematically suggest a net production of fixed carbon, i.e., net autotrophy (6, 24, 28), whereas the data from O_2 production and consumption measurements, measured in bot-

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tle incubations, indicate that the open ocean is in a net heterotrophic state (4, 33). The high temporal variability of net autotrophic episodes, in contrast with the more constant heterotrophic processes, has been suggested as a key factor that could explain this disagreement (16). However, Riser and Johnson (28) found consistent and progressive seasonal accumulation of oxygen in subsurface waters of the North Pacific subtropical gyre without the need to invoke short-lived episodes of enhanced production. In this very same region, the *in vitro* O_2 evolution method had persistently indicated a net heterotrophic metabolism in the euphotic layer throughout the year (33).

In view of the expected differential enclosure effect on auto- and heterotrophic picoplankton during bottle incubations in oligotrophic marine waters, we hypothesized that the picophytoplankton-to-heterotrophic-bacteria biomass ratio decreases during 24-h incubations in oligotrophic zones. To test this hypothesis, we measured changes in both auto- and heterotrophic picoplankton biomass during 24-h bottle incubations in the North and South Atlantic subtropical gyres. Furthermore, by sampling during a Lagrangian study following the same water mass in the middle of the north Atlantic waters, we compared the temporal changes of this biomass ratio inside the incubation bottles with those occurring *in situ*. Our results suggest that the differential bottle effect on autotrophic and heterotrophic picoplankton can help to explain the contradictory observations of the metabolic state of the oligotrophic open ocean.

MATERIALS AND METHODS

Sampling strategy and experimental setup. Experiments were conducted during TRYNITROP-2 (*Trichodesmium* and N_2 fixation in the tropical Atlantic) and CARPOS-1 (plankton-mediated carbon fluxes in contrasting subtropical oligotrophic environments: a Lagrangian approach) cruises on board the R/V *Hespérides* in April 2008 and October to November 2006, respectively. The TRYNITROP-2 cruise crossed oligotrophic waters in the North and South subtropical Atlantic, and the CARPOS-1 cruise took place in the North Atlantic subtropical gyre. A detailed description of environmental and biological conditions during these cruises can be found in the work of Marañón et al. (21) and Teira et al. (32). Our experimental and observational approach involved measurements of picoplankton biomass during (i) 24-h time series *in vitro* experiments, (ii) 24-h endpoint *in vitro* experiments, and (iii) a Lagrangian study to monitor free-living microbial populations within the same water mass over a 10-day period.

Time series experiments. We conducted three time series experiments during the TRYNITROP-2 cruise, which will be hereinafter referred to as "time series experiments." Seawater was collected, just before dawn, at two stations (E07 and E17 [Table 1]) using 15-liter acid-clean Niskin bottles attached to a rosette equipped with a conductivity-temperature-depth sensor (CTD). Water was taken from 20 m and 120 m (deep chlorophyll maximum [DCM]) at E07 and from 50 m (DCM) at E17. At each experiment, triplicate polycarbonate bottles were filled with different volumes ranging from 70 to 1,000 ml (Table 1). Prior to the experiments, bottles were thoroughly washed with 1 N HCl and then rinsed with distilled water. They were also rinsed with seawater just before filling them. Bottles were filled before sunrise with unfiltered seawater, and particular care was taken to prevent light-induced damage to the microbial populations. Time series incubations were carried out on deck, under *in situ*-simulated light (80%, 5%, and 1% photosynthetically active radiation [PAR] for surface, 50-m, and 120-m depths, respectively) and temperature conditions. Light conditions were simulated by using a combination of blue (Mist Blue; Lee Filters) and neutral density filters. The incubation temperature was maintained within 1°C of *in situ* temperature using running surface seawater for near-surface samples and recirculating water passing through a refrigerator for deep samples. The incubations lasted 24 h, and sampling for picophytoplankton and heterotrophic bacterial abundances was done on five occasions, every 3 to 6 h during the first 12 h and at 24 h. Bottles were kept in the shade during the subsampling, completely out of full sunlight.

Twenty-four-hour endpoint experiments. Sixteen 24-h incubation experiments were carried out during the CARPOS-1 cruise with water collected at 10 stations. They will be hereinafter referred to as "24-h endpoint experiments." Three additional stations were also sampled for *in situ* picoplankton abundance (see below). Table 1 shows the location and the hydrographic and biological conditions of the 13 sampling stations. Duplicate 250-ml acid-cleaned, polycarbonate bottles were filled with unfiltered surface seawater, and on six occasions, two more were filled with deeper water (Table 1). Incubations were carried out on deck, under *in situ*-simulated light and temperature conditions, as previously explained for the time series experiments. Additionally, since light conditions could affect the phytoplanktonic release of dissolved organic matter (DOM) and the heterotrophic bacterial metabolism (10), a comparison between light and dark incubations was also performed on seven occasions with surface waters and on three occasions with deeper waters in order to evaluate the effects of the light on the picoplanktonic community during the incubation experiments. Samples for picoplanktonic abundance were obtained at the beginning and the end of the 24-h endpoint experiments.

It must be stressed that, in both the time series experiments and the 24-h endpoint incubations, the procedures followed for the collection and handling of seawater samples were the same as the ones typically used during oceanographic surveys to determine *in vitro* metabolic rates of microbial plankton (9, 17).

***In situ* monitoring of microbial biomass.** Measurements of *in situ* picoplankton abundance were carried out at 13 stations during the CARPOS-1 cruise using seawater collected at surface with 15-liter acid-clean Niskin bottles. Ten of the stations sampled during the cruise were located near the center of the North Atlantic subtropical gyre (Table 1), where the same water mass was tracked by a drifting buoy during a Lagrangian study (see details in reference 32).

Hydrography, irradiance, and chlorophyll *a* concentration. Vertical profiles of temperature (from 0 to 300 m) during both cruises were obtained with a CTD SBE911 plus probe attached to a rosette equipped with Niskin bottles. Vertical profiles of photosynthetically active radiation (PAR, 400 to 700 nm) were obtained using a Satlantic OCP-100FF radiometer. Samples (250 ml) for size-fractionated chlorophyll *a* concentration (chl *a*) were filtered sequentially through 20-, 5-, 2-, and 0.2-μm polycarbonate filters and determined fluorometrically. After extraction with 90% acetone at −20°C overnight, chl *a* fluorescence was determined with a TD-700 Turner Designs fluorometer calibrated with pure chl *a*. Total chl *a* was calculated as the sum of results for all fractions.

Picoplankton abundance. Total bacterial counts were estimated from samples (1.8 ml) preserved with 1% paraformaldehyde plus 0.05% glutaraldehyde and frozen at −80°C until analysis within the same day with a FACSCalibur flow cytometer (Becton Dickinson) equipped with a laser emitting at 488 nm. Prior to analysis, heterotrophic bacteria were stained with 2.5 mM dimethyl sulfoxide (DMSO)-diluted SYBR green I DNA fluorochrome (reference no. S-7563; Molecular Probes) for 10 min in the dark at room temperature. Low- and high-nucleic-acid-content (LNA and HNA, respectively) heterotrophic bacteria were routinely distinguished based on their relative green fluorescence (FL1, 533 nm) and light scatter at 90° (side scatter [SSC]) signals. Total bacterial abundance was made up by the sum of LNA and HNA subgroups. *Prochlorococcus* counts from the unstained samples (23) were subtracted from HNA bacterial counts in surface samples due to overlapping signals. Autotrophic cells were separated *in vivo* into two groups of cyanobacteria (*Synechococcus* and *Prochlorococcus*) and one group of picoeukaryotes based on their orange (FL2, 585 nm) and red (FL3, >650 nm) fluorescence and SSC signals.

For estimating the abundance of the different groups, calibration of the cytometer flow rate was performed daily and a solution of 1-μm fluorescent latex beads (reference no. F-13081; Molecular Probes) was added as an internal standard (3). All cellular variables were related to fluorescent bead values.

Picoplankton biomass. An empirical calibration between relative SSC or forward scatter (FSC) and cell diameter, as explained by Calvo-Díaz and Morán (3), was performed on each cruise to estimate mean biovolume (BV) of picoplankton cells (Table 2). SSC or FSC were chosen on the basis of the highest variance explained in the corresponding model I linear regression. BV was finally converted into bacterial carbon biomass by using the allometric relationship of Gundersen et al. (13): bacterial biomass (fg C $cell^{-1}$) = $108.8 \times BV^{0.898}$. The following volume-to-carbon conversion factors were used for picoautotrophic groups: 230 fg C μm^{-3} for *Synechococcus*, 240 fg C μm^{-3} for *Prochlorococcus*, and 237 fg C μm^{-3} for picoeukaryotes (34).

Statistical methods. Statistical analyses were made with Statistica software (StatSoft). Ordinary least squares (OLS) linear regressions or model I was performed for examining the relationships between SSC and FSC and cell diameter (see above). For each variable, we calculated the standard error as an

TABLE 1. Initial conditions of the stations sampled during TRYNITROP-2 and CARPOS-1 cruises[a]

Station	Expt	Date (day/mo/yr)	Longitude	Latitude	Bottle vol (ml)	Temp (°C)	Chl *a* (μg liter^{-1})	1% PAR depth (m)	Depth (m)	BB (μg C liter^{-1})	picoPB (μg C liter^{-1})	%Prochl	%picoeuk
TRYNITROP-2													
E07	Time series	19/04/2008	29.00°W	9.09°S	70, 250, 1,000	28.8	0.117	121	20	2.09	3.55	72.1	26.7
									120 (DCM)	2.10	6.90	55.4	43.9
E17	Time series	29/04/2008	28.94°W	29.18°N	70, 125, 250, 1,000	20.6	0.124	123	50 (DCM)	3.89	4.60	29.9	62.8
CARPOS-1													
E04A	24h, i-s ab	18/10/2006	25.00°W	26.93°N	250	25.40	0.100	94	5	6.58	2.32	76.0	19.7
									30	6.12	3.53	73.9	22.2
E05A	24h, i-s ab	19/10/2006	29.08°W	26.39°N	250	25.77	0.165	105	5	11.09	9.01	67.3	31.2
									105 (DCM)	5.52	7.89	85.7	14.2
E06A	24h, i-s ab	20/10/2006	33.33°W	25.88°N	250	25.80	0.073	121	5	4.78	2.80	54.8	41.6
									130 (DCM)	4.39	5.70	88.6	11.4
E10 (Lagr)	24h, i-s ab	24/10/2006	36.65°W	24.85°N	250	26.78	0.108	110.43	5	5.45	4.76	55.4	37.3
E14 (Lagr)	24h, i-s ab	25/10/2006	36.64°W	24.85°N	250	26.89	0.060	113.98	5	6.78	6.63	39.0	55.9
E19 (Lagr)	i-s ab	26/10/2006	36.61°W	24.91°N		26.81	0.105	95.7	5	5.04	4.44	54.7	35.7
E26 (Lagr)	24h, i-s ab	27/10/2006	36.59°W	24.96°N	250	26.79	0.115	111.77	5	5.64	5.00	51.6	39.5
									80	5.35	8.14	85.7	11.9
E30 (Lagr)	24h, i-s ab	28/10/2006	36.57°W	25.00°N	250	26.75	0.116	116.43	5	6.06	5.05	48.4	44.1
									80	3.35	7.26	81.1	16.9
E33 (Lagr)	24h, i-s ab	29/10/2006	36.55°W	25.04°N	250	26.77	0.107	159.34	5	6.01	4.89	47.4	45.2
E37 (Lagr)	i-s ab	30/10/2006	36.52°W	25.05°N		26.71	0.108	152.76	5	5.92	6.05	46.0	45.8
E38 (Lagr)	24h, i-s ab	31/10/2006	36.52°W	25.03°N	250	26.67	0.118	146.19	5	5.70	6.90	42.8	50.6
									70	4.33	9.15	24.2	24.2
E44 (Lagr)	24h, i-s ab	01/11/2006	36.47°W	25.05°N	250	26.55	0.111	116.29	5	6.34	5.29	48.1	44.8
E47 (Lagr)	i-s ab	02/11/2006	36.41°W	25.07°N		26.42	0.114	110.43	5	6.09	5.07	48.1	44.8

[a] Initial conditions of the stations sampled during TRYNITROP-2 and CARPOS-1 cruises where the water for the time series and 24-h endpoint experiments (24h) was collected. Also noted are the stations where *in situ* picoplankton abundance was determined (i-s ab). The stations corresponding to the Lagrangian study (Lagr) are also indicated. Bottle vol, bottle volume used in each experiment; Temp, surface (5-m depth) temperature; Chl *a*, surface chlorophyll *a* concentration; BB, heterotrophic bacterial biomass; picoPB, picophytoplanktonic biomass; %Prochl, *Prochlorococcus* contribution to picophytoplankton biomass; %picoeuk, picoeukaryote contribution to picophytoplankton biomass; DCM, deep chlorophyll maximum.

TABLE 2. Linear regression models used to convert the relative signals of light side (SSC) and forward (FSC) scattering to cell diameter (μm) or volume (μm^3) of picophytoplankton and heterotrophic bacteria in each cruise

Cruise and planktonic group	Linear regression model
TRYNITROP-2	
Picophytoplankton	$\mu m = 1.2582 \times SSC + 0.4296$; $r^2 = 0.84$; $n = 9$
Heterotrophic bacteria	$\mu m^3 = 0.058 \times FSC + 0.013$; $r^2 = 0.60$; $n = 13$
CARPOS-1	
Cyanobacteria	$\mu m = 1.0049 \times SSC + 0.6297$; $r^2 = 0.47$; $n = 10$
Picoeukaryotes	$\mu m = 0.5389 \times SSC + 0.753$; $r^2 = 0.87$; $n = 17$
Heterotrophic bacteria	$\mu m = 0.7871 \times SSC + 0.4654$; $r^2 = 0.60$; $n = 16$

indicator of uncertainty in our measurements. The Student *t* test for comparisons of means was applied to detect differences for each variable between the beginning and the end of the incubations of both time series and 24-h endpoint experiments.

RESULTS AND DISCUSSION

All of the stations were located in oligotrophic waters of the central Atlantic Ocean, characterized by warm temperatures at the surface (>25°C), strong thermal stratification, and low concentrations of both inorganic nutrient and chl *a* in the upper mixed layer. Initial conditions for each experiment and sampling stations are presented in Table 1. The euphotic layer (1% PAR level) was deeper at the center of the North Atlantic gyre, where it reached a 159-m depth. Surface chl *a* ranged from 0.06 to 0.12 mg m^{-3} during the initial conditions of the experiments. More information about initial hydrographic and biological conditions can be found in work by Marañón et al. (21) and Teira et al. (32).

Time series experiments. We observed conspicuous changes in autotrophic biomass during incubations, with contrasting trends for each picophytoplanktonic group (Fig. 1). A marked linear decrease of *Prochlorococcus* biomass was found during the first 6 h of the time series experiments regardless of bottle volume, with a biomass decrease ranging from 38 to 73% (Fig. 1D to F). *Prochlorococcus* biomass after 24 h was significantly lower than that observed at the initial time for all the time series experiments (paired *t* test, $P < 0.001$, $n = 10$). However, the evolution of *Synechococcus* biomass was irregular, lacking any obvious general effect. This genus showed a clear decrease in only one of the time series experiments (Fig. 1B), with rather stable biomass, or even a slight increase before 6 to 12 h, observed in the other two experiments (Fig. 1A and C). If we consider all the experiments and treatments together, these changes were not statistically significant in terms of biomass after 24-h incubations (paired *t* test, $P = 0.17$, $n = 10$). The different responses displayed by the two genera of cyanobacteria show that although they are likely subject to similar controls, they respond differently to biological (e.g., grazing) and physicochemical (light, nutrients) factors. *Prochlorococcus* has been found to be particularly vulnerable to the stress from handling, confinement, and/or solar radiation exposure compared to *Synechococcus* (19). These factors, connected with a differential grazing pressure on the two genera of cyanobacteria (reference 34 and references therein), could partly explain the differences observed during incubations.

The biomass of picoeukaryotes decreased in all the experiments (Fig. 1G to I). After 24 h, the biomass decline ranged from 17 to 85%, and biomass was significantly lower than that at the beginning of the experiments (paired *t* test, $P < 0.001$; $n = 10$), suggesting picoeukaryotes to be impaired. In contrast, the biomass of total heterotrophic bacteria doubled at station E07 (Fig. 1J and K) and it was relatively stable during the experiment at E17 (Fig. 1L). Confinement may give rise to trophic cascade effects, involving changes in the composition of micro- and nanoplanktonic heterotrophic protists, thus changing the grazing pressure experienced by the picoplanktonic phyto- and bacterioplankton. This effect may take place regardless of the sample volume, since volumes of incubation bottles did not affect the observed trends of the picoplanktonic groups. Confinement also prevents exchange of nutrients with

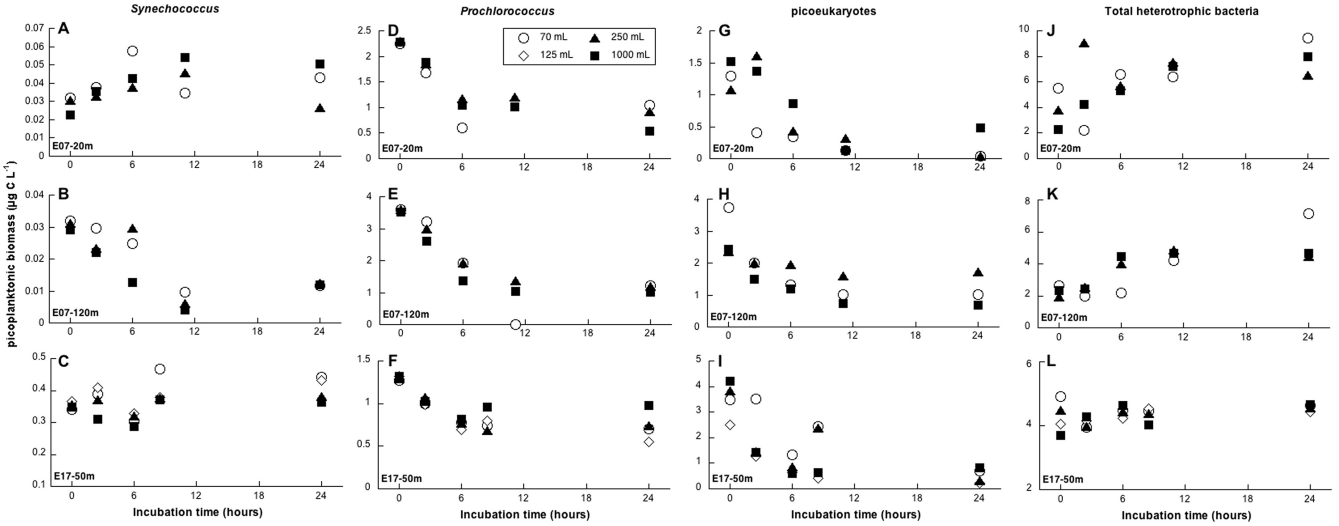

FIG. 1. Changes in the biomass of picoplankton groups during the time series experiments in bottles of different volumes at stations E07 (29.00°W, 9.09°S) and E17 (28.94°W, 29.18°N). (A to C) *Synechococcus*; (D to F) *Prochlorococcus*; (G to I) picoeukaryotes; (J to L) heterotrophic bacteria. Note different scales on the *y* axes.

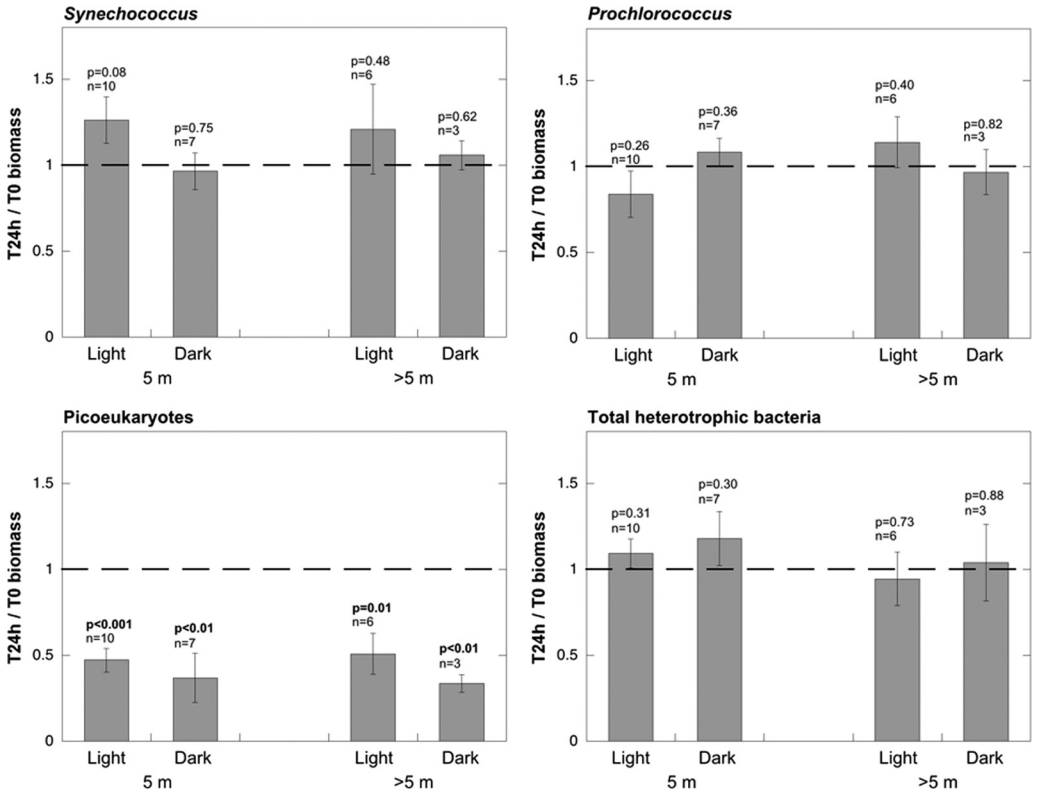

FIG. 2. Average (± standard error [SE]) of the ratio, calculated for the 24-h endpoint experiments, between biomass after 24 h of incubation (T_{24h}) and at initial conditions (T_0) for picophytoplanktonic groups and total heterotrophic bacteria in surface (5-m) and deeper (>5-m) waters. Results from light and dark incubations are shown.

surrounding water. Consequently, a decrease of nutrient concentration is expected during the incubations. Since picoeukaryotes have higher nutrient requirements than smaller picophytoplanktonic cells (i.e., cyanobacteria) (27), this limitation of picoeukaryotic growth rate by nutrient supply and a stronger grazing pressure, possibly induced by a smaller presence of predators feeding on heterotrophic nanoplankton, may have given way to the observed dramatic decreasing trend upon confinement.

Twenty-four-hour endpoint experiments. In contrast to what we found in the time series experiments, the biomass of *Prochlorococcus* did not change significantly in 24-h endpoint experiments with both surface (5-m) and deeper (>5-m) waters. The ratio between biomass at 24 h and biomass at 0 h (T_{24h}/T_0 biomass) was not significantly different from 1 (Fig. 2). However, the biomass of picoeukaryotes was always significantly lower after 24 h in both surface and deeper waters (Fig. 2), as found during the time series experiments (Fig. 1). Differences in biomass between light and dark conditions were not found in 24-h incubations (paired *t* test, $P > 0.05$; $n = 7$ for surface waters and $n = 3$ for deeper waters), indicating that light conditions were not directly responsible for changes in the biomass of the picoplanktonic groups assessed during the experiments. Picoeukaryotic contribution to total picoautotrophic biomass averaged 41% ± 3% at the surface and 17% ± 2% in deeper waters. Despite their low abundance, compared to that of picoplanktonic cyanobacteria, the picoeukaryotes are major contributors to total primary production in oligotrophic waters because of their larger cell volume and higher cell-specific carbon fixation rates (15). Thus, the consistent decrease of picoeukaryotic biomass found in time series experiments and 24-h endpoint experiments implies a serious underestimation of ocean productivity.

Heterotrophic bacteria showed a trend to increase slightly their biomass after 24 h in most of the experiments (Fig. 2). Previous works have shown an exponential increase in numbers of marine bacteria during 24-h incubations (26), but relatively constant biomass during 24-h incubations has also been reported (11). The overall effect of bottle confinement could be the consequence of multiple processes, including artificial enrichment of substrates resulting from phytoplankton cell death, effects of interfaces (walls) on bacterial activity, appearance of trophic cascades, and changes in initial microbial compositions (7).

AB:HB ratio. Most methods employed to determine metabolic rates related to production and consumption of organic matter use discrete incubations of the *in situ* community. If we want to compare only either autotrophic or heterotrophic processes between different regions or seasons (for instance, only primary production or only bacterial production, protistan grazing, or community respiration), we can assume that a similar degree of error is shared by all spatial or temporal measurements. However, if the results obtained are used to estimate net community production, a key variable determining the role of the biota in carbon cycling, the autotrophic and heterotrophic components must be considered simultaneously.

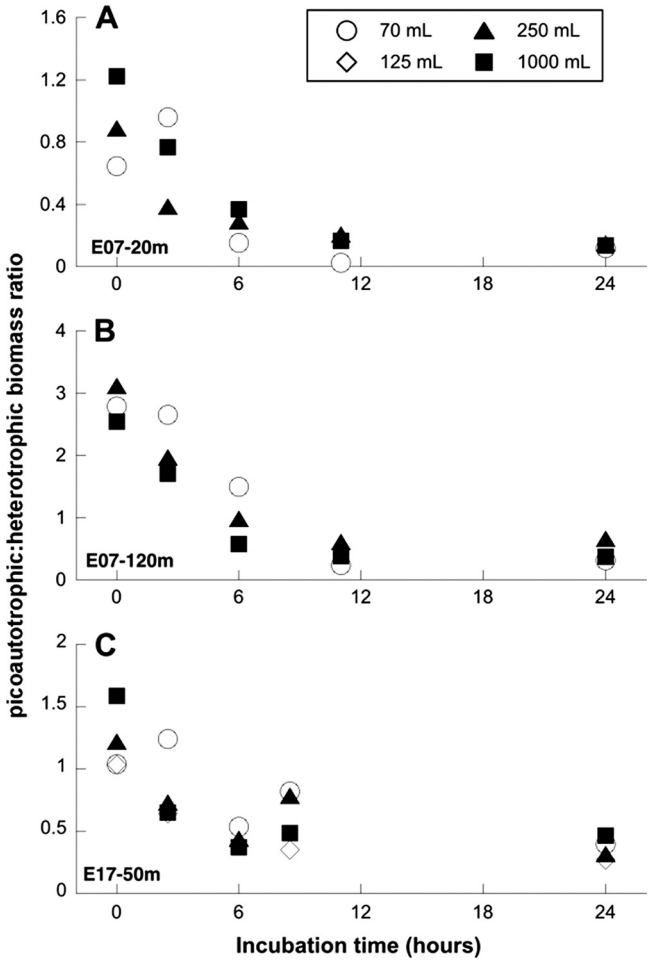

FIG. 3. Temporal evolution of the autotrophic to heterotrophic biomass ratio in picoplankton during the time series experiments at stations E07 (29.00°W, 9.09°S) and E17 (28.94°W, 29.18°N). Data for bottles of different volumes are shown.

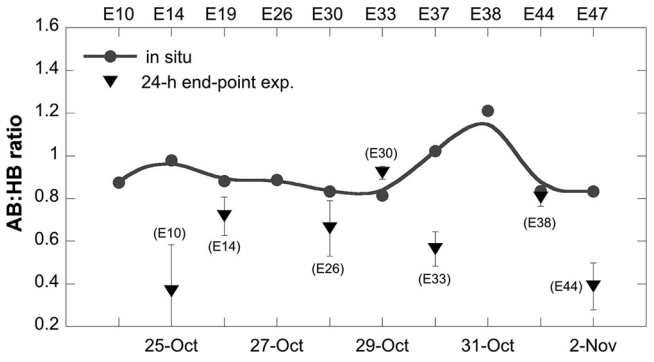

FIG. 4. Temporal evolution of the autotrophic to heterotrophic biomass ratio (AB:HB) in surface picoplankton of the same water mass during the Lagrangian study (from 36.65°W, 24.85°N to 36.41°W, 25.07°N; October to November 2006). Sampling was carried out every 24 h. Surface AB:HB ratio (±SE as error bars) at the end of 24-h endpoint bottle incubations (24-h end-point exp.) is also shown at the corresponding date. The location (station number) where the water was collected for the experiments is shown in parentheses.

In this regard, it is critical to assess if confinement differentially affects the autotrophic versus heterotrophic microorganisms. In oligotrophic marine environments, most primary production is due to small-sized phytoplankton (15, 25, 31) and bacteria contribute a major fraction of total microbial respiration (29). Thus, it is important to analyze the changes in the ratio of autotrophic to heterotrophic biomass (AB:HB ratio) in the picoplankton size class during bottle incubations.

The AB:HB ratio markedly decreased in all the time series experiments (Fig. 3). This effect was larger in the first 6 to 12 h, in which 77% decreases (range: 48 to 96%) were observed, remaining relatively constant during the rest of the incubation, with an average decrease of 79% ± 3% after 24 h. This finding could be interpreted as indicating that bottle confinement affects in a different way the autotrophic and heterotrophic picoplankton groups. However, the possibility exists that these changes reflect natural variability over diurnal time scales, which would imply that the same decreasing pattern would be observed *in situ*.

Daily sampling during the Lagrangian study near the center of the oligotrophic gyre (Table 1) enabled us to track the *in situ* temporal changes of the picoplankton community within the same water mass and, therefore, to compare *in situ* changes with those occurring inside the incubation bottles. Figure 4 shows the relatively constant values of the *in situ* surface AB:HB ratio found during the Lagrangian study. Values ranged from 0.81 to 1.21 but, for most of the time, gathered around 0.85, with a short-lived increase close to the end of the sampling period. Seven 24-h endpoint experiments were performed during this period (E10, E14, E26, E30, E33, E38, E44 [Table 1]), and the surface AB:HB ratio after 24-h incubation was, in all except one of the bottles (E30), lower than that found *in situ* the next day (Fig. 4). These differences were significant (paired t test, $P < 0.05$; $n = 7$), and the mean AB:HB ratio in the bottles was 29% lower than the mean value found *in situ*. In addition, the AB:HB ratio found *in vitro* was more variable than that observed *in situ* (coefficient of variation [CV], 33% versus 13%).

Further, we calculated a ratio ($AB:HB_{24-0}$) between the AB:HB ratio after 24-h incubation and the AB:HB ratio at the initial time of incubation in all light and dark 24-h endpoint experiments performed with surface water in the North Atlantic subtropical gyre. We also calculated the same ratio ($AB:HB_{24-0}$) for the *in situ* samples during the Lagrangian study (since the frequency of the *in situ* sampling was 24 h). A value of 1 would mean that the AB:HB ratio did not change after 24-h bottle incubations or after 24 h under *in situ* conditions. On the other hand, a ratio different from 1 would mean an increase or a decrease of the AB:HB ratio. This comparison between ratios from experiments versus *in situ* samples thus allows us to detect bottle confinement effects. As expected for surface oligotrophic waters, characterized by relatively stable environmental conditions over time scales of a few days, we did not observe significant changes in the $AB:HB_{24-0}$ ratio under *in situ* conditions (Fig. 5). In contrast, after 24-h bottle incubations, the $AB:HB_{24-0}$ ratio was significantly lower than 1 for surface samples under both light and dark conditions (Fig. 5). These results confirmed that whereas the biomass of autotrophic and heterotrophic microbial compartments and their interaction were rather constant under *in situ* conditions over

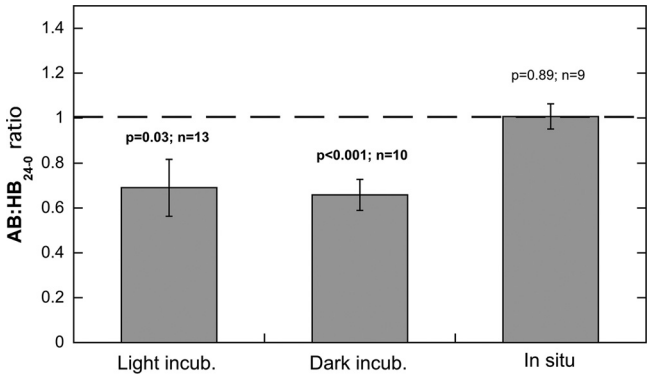

FIG. 5. Average (±standard error [SE]) of the ratio between AB:HB after 24 h of incubation and AB:HB at initial time of the incubation ($AB:HB_{24-0}$ ratio) from surface waters under light (Light incub.) and dark (Dark incub.) incubation conditions. This ratio between two subsequent Lagrangian sampling stations (24 h of frequency sampling) for *in situ* samples (In situ) is also shown.

short time periods in oligotrophic conditions, there was a significant effect of bottle enclosure on picoplanktonic groups and this effect was consistently different for the autotrophic and heterotrophic picoplankton.

Conclusions and implications. Our results from oligotrophic waters of the Atlantic Ocean indicate that in 24-h bottle enclosure experiments, typically designed to measure physiological and functional properties of picoplanktonic communities, there are frequently divergent biomass responses of autotrophic and heterotrophic components, which do not take place in natural conditions *in situ*. These responses consist of a decrease in picoautotrophic biomass, typically affecting the picoeukaryotes but often involving also *Prochlorococcus*, and a less marked increase in the biomass of heterotrophic bacteria. The appearance of trophic cascades and the expected decrease in nutrient concentrations during the incubation, involving changes in the structure of the planktonic community, could be the main causes for the observed effects of bottle confinement. If the relative importance of autotrophic and heterotrophic biomass quickly changes in a persistent and predictable way inside the bottles, this is likely to affect the final estimate of metabolic balances. Given that the predominantly observed response is a decrease in the picophytoplankton to heterotrophic bacteria biomass ratio, an expected consequence is that bottle incubations will tend to overestimate the respiration to photosynthesis ratio. This may occur through two mechanisms that can operate separately or in conjunction: an impairment of picophytoplankton photosynthesis and a stimulation of bacterial metabolism and, hence, bacterial respiration. This artifact may partially explain the discrepancy, regarding the metabolic status of the oligotrophic ocean, between studies using *in vitro* O_2 evolution measurements (4, 33) and biogeochemical approaches, such as the monitoring of *in situ* O_2 accumulation (28). In the light of these results, we strongly advise checking the changes in the relative biomass of picophytoplankton and heterotrophic bacterioplankton during bottle incubations of oligotrophic marine waters.

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